The effects of increased atmospheric reactive nitrogen deposition upon rates of biological nitrogen fixation in peatlands and temperate forests

Ernesto Saiz Val

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Abstract

Biological nitrogen fixation (BNF) is a microbial process that fixes atmospheric nitrogen (N_2) gas into reactive, bioavailable forms (Nr). Naturally, lightning and BNF accounted for the entire Nr available for plant and microbial metabolic demands. However, following industrialization, Nr has also been produced anthropogenically (e.g. burning fossil fuel, fertilizers). The UK experiences high rates of anthropogenic Nr deposition (>26 kg $ha^{-1}yr^{-1}$) not being clear if increased Nr deposition slows or shuts down BNF in peatlands and temperate forests, as Nr is freely available. Therefore, it was critical to investigate it. The research objectives were to establish a robust method to measure BNF, to evaluate the impact of chronic Nr deposition on BNF and to examine the main factors controlling BNF. Following comparative evaluation of the two common methods for quantifying BNF in peatlands, the acetylene reduction assay underestimated BNF by 53% compared to the direct $^{15}N_2$ assimilation method. Across a gradient of Nr deposition, higher rates of *in situ* BNF were found in areas with lower Nr deposition rates and BNF decreased as Nr increased; however, BNF did not shut down completely. Under experimental long-term addition of Nr and sulphur into a peatland in northern Sweden, BNF decreased by 94%. *Sphagnum* mosses exposed to higher Nr deposition exhibited an increase in BNF by 83% following the addition of P and K. Addition of microbial respiratory metabolites (CH_4, CO_2 and N_2O) to mosses enhanced BNF 80%. Asymbiotic BNF in deciduous temperate forest soils exposed to ~22 kg Nr annual deposition increased by 368% under elevated CO_2 fumigation. This research demonstrates that BNF does not shut down under chronic atmospheric Nr and that increased availability of nutrients and energy for microbes can further boost BNF. Therefore, BNF must be considered even in polluted areas when modelling the N economy of their ecosystems.

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CHAPTER 1: INTRODUCTION

1.1 Peatlands, temperate forests, and the nitrogen cycle

1.1.1 Peatlands

Wetlands including peatlands in the broadest term are characterized by the presence of water saturation of the surface layers at least during the growing season that results in anoxic conditions. Such conditions then support plant species which are adapted to wet and complete and/or partial anoxic and wet conditions. Peatlands are a type of wetland in which under these wet conditions the remains of plants and animal constituents accumulate partially decomposed, i.e. forming peat (Mitsch and Gosselink, 1993). A peatland to be defined as such needs to accumulate at least 30 cm of peat. Peat, thus, is the result of a set of conditions that are met in peatlands: accumulation of different plant materials (e.g. leaves, woody parts, bryophytes - especially *Sphagnum* mosses) from above ground that is difficult to decompose, some belowground plant parts (e.g. roots) and invertebrates that accumulate as well to the peat, anoxic conditions due to water saturation, and specific biogeochemistry (Rydin and Jeglum, 2013). Wetlands comprise about the 3% of the world's land surface and are located mostly in the temperate zone of the northern hemisphere (Moore, 2002), in fact, peatlands, a type of wetlands, cover about 10 % of the United Kingdom's land surface (Montanarella et al., 2006). Peatlands can be divided conventionally into two different types: fens and bogs (Moore, 2002).

Peatlands are mainly divided into minerotrophic and ombrotrophic as they are flow-fed or rain-fed respectively. Fens are minerotrophic peatlands that have the water table above the surface, at, or a bit below (Rydin and Jeglum, 2006). They receive some of the nutrients from the surrounding mineral substrate, apart from the ones received from rainfall (Lindsay, 1995; Kivimaki, 2011). Mineral soils have the capacity to neutralize the acidity that some processes such as cation exchange or respiration produce, because of this, pH in fens is

ranging approximately from 5 to 8 (Davis and Anderson, 1991). The vegetation that often covers these areas is formed usually by sedges, reeds, or grasses (Mitsch and Gosselink, 1993). Although *Sphagnum* mosses may be present in rich fens (high pH and electrical conductivity), the common ones in these areas are the denominated brown mosses (e.g. *Calliergon* spp., *Scorpidium* spp., *Campylium stellatum*, *Calliergonella cuspidata*; Wheeler and Proctor, 2000). Other common species in fens are *Carex* spp. (sedges) and *Molinia caerulea* (grasses; Øien and Moen, 2001). Regarding herbs a high diversity can be usually found in rich fens with species such as *Cypripedium calceolus*, *Liparis loeselii*, *Pinguicula vulgaris*, *Rubus chamaemorus*, *Menyanthes trifoliate*, etc. (Rydin and Jeglum, 2013).

Bogs are ombrotrophic peatlands that receive nutrients only from direct atmospheric deposition (Davis and Anderson, 1991; Lindsay, 1995; Rydin and Jeglum, 2006). The low amount of nutrients that reach the ombrotrophic bogs makes them oligotrophic (infertile, poorly-fed), and also facilitates the increase of acidity (Davis and Anderson, 1991). In Britain, one of the most acidic and nutrient-poor environments is represented by ombrotrophic bogs (Lindsay, 1995). Due to this fact, only a very limited number of adapted plant species can live in these nutrient-poor and acidic conditions. This kind of peatlands are usually dominated by *Sphagnum* mosses (peat moss), because they are adapted to nutrient-poor and low pH environments. Other bryophytes that can also be found in temperate bogs are *Rhytidiadelphus loreus* (feather moss), *Cladonia* spp (moss-like lichen), and *Racomitrium lanuginosum* (Rydin and Jeglum, 2013). Graminoids and herbs are not diverse in bogs, but *Eriophorum vaginatum* and *Eriophorum angustifolium* (cotton grass) and *Nardus* spp. are abundant, and other species such as *Potentilla erecta* can also be found (Schillereff et al., 2016). Other typical vegetation for bogs are shrubs, particularly evergreen dwarf ones such as *Calluna Vulgaris*, *Erica tetralix*, and *Vaccinium* spp. (Schillereff et al., 2016). They are common in cool temperate zones and primarily in northern Europe in the

boreal and northern deciduous biomes (Mitsch and Gosselink, 1993). Different kind of trees can grow in this type of peatland such as conifer in norther continental regions; or deciduous in more southern areas; but in oceanic regions bogs usually are not forested and dominated by *Sphagnum* mosses (Moore, 2002).

The importance of hydrology in peatlands is paramount as water is their main feature. There are a set of factors that define their hydrologic state which are the surface of the landscape, the subsurface characteristics (geology, soil, and groundwater), and the difference between water inflows and outflows (Mitsch and Gosselink, 1993). The water budget of a peatland is expressed considering the amount of water inputs, outputs, and storage as in the following equation (after Gilvear and Bradley, 2000; see also Fig. 1.1):

$$P_n + Q_{in} + G_{in} = E + Q_{out} + G_{out} \pm S \qquad \text{(Equation 1-1)}$$

where P_n is net precipitation; Q_{in} is surface inflow; G_{in} is groundwater inflow; *E* is evapotranspiration; Q_{out} is surface outflow; G_{out} is groundwater outflow; S is water storage.

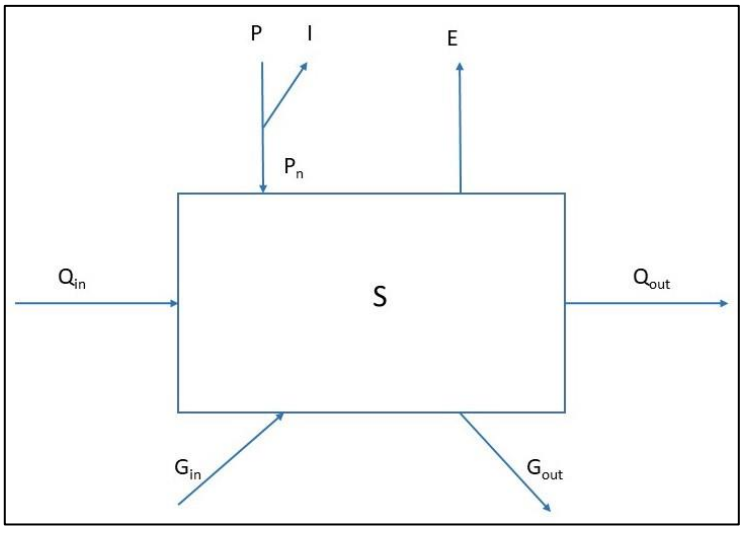


Figure 1.1. General water budget for a peatland. It corresponds to equation 1-1. S = water storage; P_n = net precipitation (precipitation (P) – interception (I)); Q_{in} = surface inflow; G_{in} = groundwater inflow; E = evapotranspiration; Q_{out} = surface outflow; G_{out} = groundwater outflow. (Source: after Mitsch and Gosselink, 1993)

Not all the terms apply for all the types of peatlands, and in the same peatland, it could happen that the terms that apply could be different between years. An example of the latter can be observed in Figure 1.2 a) where the second year there was groundwater outflow but not the first year. The water storage can also vary between years reaching differences of up to 87% as Figure 1.2 b) shows. In both types of peatlands represented in Figure 1.2 gains are in excess of losses which is essential for peatlands to occur (Mitsch and Gosselink, 1993). A study on peatland hydrology suggests that even changes in the precipitation frequency may alter peatlands biogeochemistry and vegetation communities, highlighting the effects of climate change (Radu and Duval, 2018).

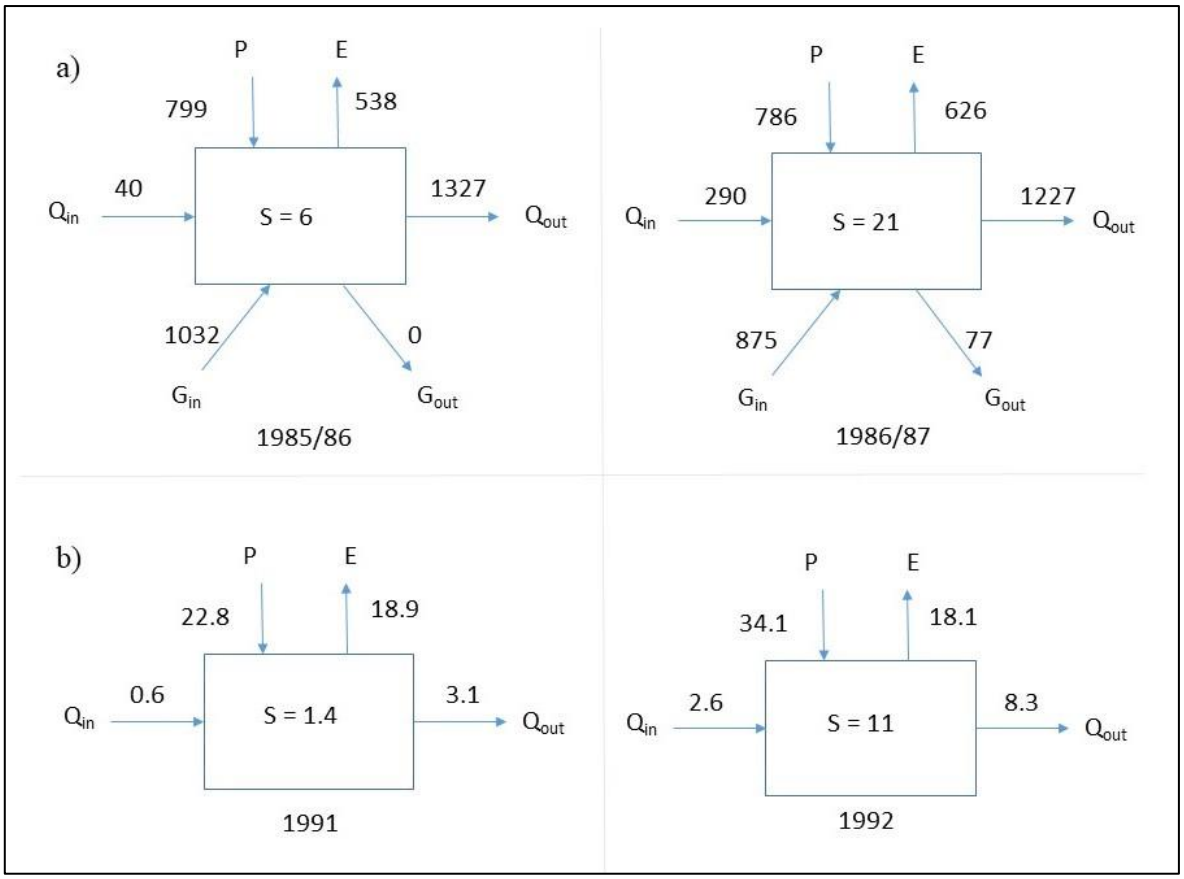


Figure 1.2. Annual water budget for several peatlands for two consecutive years (12 months): a) a fen in the Netherlands (data in mm; after Koerselman, 1989); and b) a bog in England (data in 10^3 m^3 after Bradley, 1997).

Although peatlands cover less than 3% of the world´s land surface, they store about 25% of the Earth's soil carbon (C; Yu, 2012) and 9 to 16% of the Earth's soil nitrogen (N; Limpens et al., 2006). This storage capacity relies on the net primary productivity (NPP) of peatlands that exceed decomposition rates, and that they have almost no N gas fluxes, small N losses through runoff, and low N mineralization rates (Vile et al., 2014), thus making these ecosystems relatively tightly coupled with reference to N cycling.

1.1.2 Temperate forests

Forest is usually defined in dictionaries as a "large area of land full of trees and plants". Globally, forests cover about 30% of the Earth's land surface becoming one of the most important and largest ecosystems in the world (Llado et al., 2017). Temperate forests can be found between the Arctic Circle and the Tropic of Cancer in the northern hemisphere and the Tropic of Capricorn and the Antarctic Circle in the southern hemisphere (Monson, 2014), however there is no line to separate biomes, but a gradual transition to boreal forests where conifers start to dominate closer to the poles, or to subtropical biomes (e.g. rainforests) closer to the equator. Temperate forests occur more extensively in the northern hemisphere and one of their main characteristics is seasonality, with well-defined cold winters and hot summers and temperatures ranging from -30 ºC to 30ºC respectively (Llado et al., 2017). They have an annual precipitation range between 750 and 1500 mm being the second rainiest biome after rainforests (McCarragher and Rigg, 2016). Within the temperate forest latitude band, the occurrence of other forest types at the landscape scale is due to other factors such as soil type, nutrient availability, substrate, elevation, and drainage (Llado et al., 2017).

In the northern hemisphere, temperate forests are mainly dominated by a variety of deciduous trees. Five different layers can be differentiated. First, tall canopy trees, up to 30 m, such as oak (*Quercus* spp.), sycamore (*Acer* spp.), or silver birch (*Betula* spp.) trees. The next layer is the subcanopy which is made up of saplings and other small trees such as holly (*Ilex* spp.) or hazel (*Corylus* spp.). Then there is a shrub layer where dogwood (*Cornus* spp.), huckleberries (*Gaylussacia* spp.), or viburnum (*Viburnum* spp.) specimens, among many others, could be found. Below all the previous layers appears the herbaceous one that is composed by non-woody plants such as grasses, wildflowers, and ferns. And finally, on the forest floor and covering trunks and woody debris lichens, e.g. *Arthonia* spp., and mosses, e.g. *Eurhynchium striatum* or *Hypnum cupressiforme*, form a blanket, although it may depend fundamentally on moisture (McCarragher and Rigg, 2016).

Temperate forests play a crucial role as carbon sinks. In fact, 861 Pg is the estimation of C stock stored globally in forests, of which the highest percentages are in soil (44%) and in above and belowground biomass (42%). Of the global stock of C, 14% is stored in temperate forests (Llado et al. 2017), even though they are the most exploited and degraded forests in the world due to numerous human activities such as urbanization, clearing for agriculture, or unsustainable harvesting (McCarragher and Rigg, 2016). The cycle of C is closely linked to the cycling of N as the latter controls different processes of the ecosystem (Vile et al., 2014). N is one of the most abundant elements in the Earth, however it is found mainly in the atmosphere (78%) in the form of N_2 and it is not available to plants, except for the ones with the capacity of breaking the triple bond through the N fixation process. As a result of the high N requirements by plants and the low availability, plant's growth can be limited by N, particularly in terrestrial ecosystems as forests (Son, 2001).

1.1.3 The nitrogen cycle

The fifth most abundant element in the solar system is N (Canfield et al., 2010). This element is essential for life, and although it is the most abundant in the atmosphere (78%), it is not bioavailable. It cannot be used by the majority of the organisms because of the high amount of energy that is required to break the triple covalent chemical bond of the N_2 molecule (Dodds, 2002). Nevertheless, some organisms are specialised in the transformation of N_2 into "reactive forms" of nitrogen (bioavailable forms), and this process is called biological nitrogen fixation (BNF). Other natural form of N fixation is lightning. The amount of energy generated is enough to trigger the combination of N_2 and O_2 to form NO_3^- (Dodds, 2002). Reactive nitrogen (Nr) was generated only by BNF and lightning prior to human intervention in the N cycle (Fowler et al., 2013). Galloway et al. (2003) affirm that with no humans the environment did not accumulate Nr because the processes in both ways (BNF and denitrification) were compensated. However, Stein and Klotz (2016) state that since the Haber-Bosh industrial process started in 1909 (to transform N_2 into ammonia) the amount of Nr created exceeds the demand, generating an important degradation of the environment. Table 1.1 shows how by the 2000s the proportion of the anthropogenic contribution to the Nr creation was almost 50% of the total Nr creation and how, while in 1860 there was no Nr excess (simplifying Nr creation vs denitrification) by the 2000 the Nr excess was 110 Tg N yr^{-1}. In addition, Table 1.1 also reveals an important increase in atmospheric Nr emission and deposition by more than 70% in both cases of the reduced (NHx) and oxidised (NOx) forms. This increase in atmospheric Nr deposition pose a threat to BNF and to ecosystems such as peatlands, and it modifies the global N cycle.

Table 1.1. Main Nr fluxes in the global N cycle, in Tg N yr^{-1}. (1860 data from Galloway et al., 2004; 2000s data from Fowler et al., 2013)

	1860	**2000s**
Nr creation		
Natural		
Lightning	5.4	5
BNF-terrestrial	120	58
BNF-marine	121	140
Natural total	*246.4*	*203*
Anthropogenic		
Haber-Bosch	0	120
BNF-cultivation	15	60
Fossil fuel combustion	0.3	30
Anthropogenic total	*15.3*	*210*
Total Nr creation	***262***	***413***
% Anthropogenic Nr creation	***5.84%***	***49.15%***
Atmospheric emission		
NOx		
Fossil fuel combustion	0.3	30
Lightning	5.4	5
Other emissions	7.4	10
NH_3		
Terrestrial	12.9	60
Marine	5.6	9
N_2O		
Terrestrial	8.1	13
Marine	3.9	5.5
Total NOx and NH_3	***31.6***	***114***
Atmospheric deposition		
NOx		
Terrestrial	6.6	30
Marine	6.2	22
NOy total	*12.8*	*52*
NHx		
Terrestrial	10.8	42
Marine	8	17
NHx total	*18.8*	*59*
Total atmospheric deposition	***32***	***111***
Denitrification		
Continental	98	113
Oceanic	172	190
Total denitrification	***270***	***303***

N is an element that is very mobile, which means that has low residence times travelling between the different stages of the N cycle that are the atmosphere, the living organisms, and the soil, and it is the element with most complex interactions in the processes (Rydin

and Jeglum, 2006). There are nine nitrogen molecules (Table 1.2) that represent the different oxidation states of the nitrogen, of which the most important in terrestrial ecosystems, e.g. peatlands and forests, are the inorganic forms nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+), that are the bioavailable forms of N (Dodds, 2002). Other N forms that are critical in these ecosystems are nitrous oxide (N_2O), as a resultant gas of a microbial process, and dinitrogen (N_2) gas, which can be the result of or the source of different microbial processes. Naturally, particularly in wet soils, N is the most limiting nutrient (Mitsch and Gosselink, 1993).

Table 1.2. Different N-cycle molecules representing the nine oxidation states. (After Stein and Klotz, 2016)

Molecule	**Name**	**Oxidation state**	
NH_3, NH_4^+	Ammonia, ammonium	-3	Reduced
N_2H_4	Hydrazine	-2	↑
NH_2OH	Hydroxylamine	-1	↑
N_2	Dinitrogen	0	↑
N_2O	Nitrous oxide	+1	↑
NO	Nitric oxide	+2	↑
NO_2^-	Nitrite	+3	↑
NO_2	Nitrogen dioxide	+4	↑
NO_3^-	Nitrate	+5	Oxidized

In terrestrial ecosystems such as peatlands and forests, the main pool of N is present in dead organic matter in soils where internal fluxes take place and from which are measured inputs and outputs (Limpens et al., 2006; Esser et al., 2011). N movements have conventionally been divided into inputs to the ecosystem (BNF, lighting, volcanoes), and losses (denitrification, industrial combustion, biomass burning, and ocean burial; Ward, 2012). However, the nitrogen cycle comprises many flows with different biogeochemical processes (Fig. 1.3) and the major ones are: BNF that is the focus of this work, plant uptake (lock-up of N in plant tissues), decomposition/mineralization, immobilization (lock-up of N in

microbes), nitrification, dissimilatory nitrate reduction to ammonia (DNRA), denitrification, and anaerobic ammonium oxidation (anammox).

N_2 fixation, generally known as BNF, is the conversion of N_2 gas, via its reduction, to NH_3 following the equation (Postgate, 1982):

$$N_2 + 8H^+ + 6e^- \rightarrow 2NH_3 + H_2 \qquad \text{(Equation 1-2)}$$

Only some microorganisms have the capacity to fix N_2, which are those with the enzyme nitrogenase. In N-limited environments such as peatlands and forests, the most common N-fixing organisms (diazotrophic organisms) are archaea and free-living, symbiotic and associative bacteria (Knorr et al., 2015). The vegetation that dominates northern peatlands are *Sphagnum* mosses, which are colonized by different N_2 fixers such as cyanobacteria, and play a key role in the N cycle (Leppänen et al., 2015). The BNF process may happen in different particular environments of peatlands and forests such as the aerobic peat layer or top organic soil, the anaerobic peat layer, the top waters, the rhizosphere of the plants, the stem and leaves parts of the plant (Mitsch and Gosselink, 1993). Moreover, the fixation process is favoured by anoxic conditions because oxygen inhibits nitrogenase activity, however it can be carried out in aerobic conditions but protecting nitrogenase from oxygen exposure (Dodds, 2002).

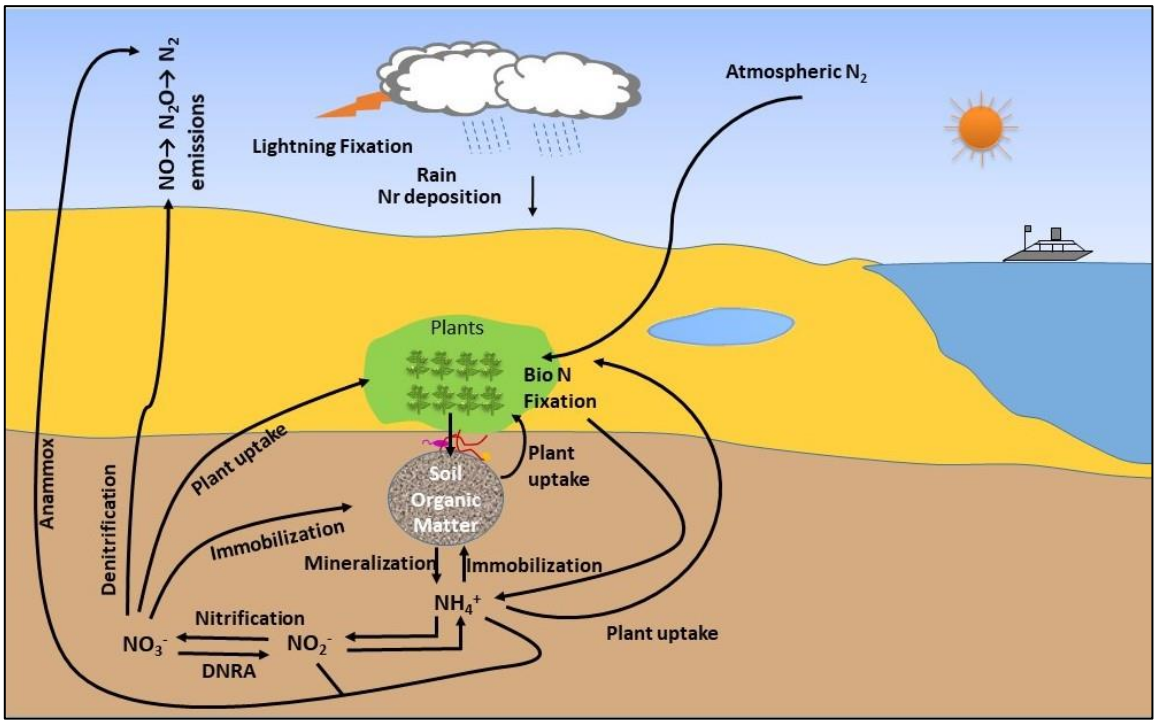
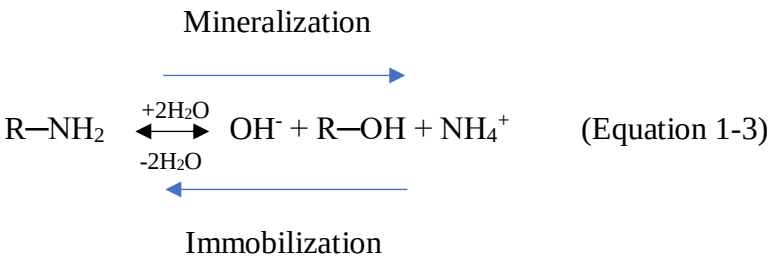


Figure 1.3. N cycle main processes in terrestrial ecosystems. (Source: prepared by the author)

Plant uptake is the removal of the bioavailable forms of N, NO_3^- and NH_4^+, by plants. Under some conditions, plants may also take up directly some amino acids (N_{org}; Weil and Brady, 2017).

Mineralization refers to the decomposition of organic N from organic matter by microbes that transform it into ammonium. This process can happen in both oxic and anoxic conditions, and it is performed by the decomposers of organic matter, heterotrophic bacteria and fungi, but in bogs, due to the low pH, fungi are more important for this process (Rydin and Jeglum, 2006). This mineralization process usually takes two steps: the first one where heterotrophic bacteria break complex organic compounds into more simple amino acids and amines; and the second one where other group of heterotrophs hydrolyse the resulted amine groups releasing NH_4^+ (McGrath et al., 2014). This last step may be represented as follows,

where the amino compound is used to indicate the source of organic nitrogen (Weil and Brady, 2017):



(Equation 1-3)

Williams and Sparling (1988) found that mineralization was highly correlated with microbial biomass. Bogs are often very acidic environments so that they have a very low bacterial activity, and thus very low mineralization rates (so they keep a pool of N_{org}) which contrast with the high ones in forests. In addition, *Sphagnum* moss, the dominant vegetation in bogs, is not easy to degrade, and low nitrogen abundance may limit the decomposition process (Rydin and Jeglum, 2006). In fact, some authors have found that the increase in Nr deposition may increase the rates of decomposition, as well as the rates of nitrous oxide emission, mineralization and denitrification, and thus may alter the nitrogen cycle (Verhoeven et al., 1996).

Immobilization occurs when bioavailable inorganic molecules of N (NO_3^- and NH_4^+) are transformed into organic forms (R-NH_2). This process is done mostly by microorganisms when decomposing organic matter, but occasionally it can take place through abiotic processes, which could be quite important in forested soils (Weil and Brady, 2017). This way microorganisms lock up the N temporarily (immobilize), i.e. it is not available for plants until they die and the N_{org} is released and transformed into the bioavailable forms.

Nitrification is the process by which some bacteria get energy through the oxidation of NH_4^+ to NO_2^- in a first stage (by e.g. Nitrosomonas), and then to NO_3^- (by e.g. Nitrobacter) in a second stage which can be expressed in a simple way as follows (Weil and Brady, 2017):

Step 1:

$$NH_4^+ + 1½O_2 \xrightarrow[\text{bacteria}]{\textit{Nitrosomas}} NO_2^- + 2H^+ + H_2O + 275\ \text{kJ energy} \quad \text{(Equation 1-4)}$$

Step 2:

$$NO_2\text{-} + ½O_2 \xrightarrow[\text{bacteria}]{\textit{Nitrobacter}} NO_3^- + 76\ \text{kJ energy} \quad \text{(Equation 1-5)}$$

In anoxic environments, it cannot take place because it is not energy effective, the main objective of the process, which is dissimilatory as far as nitrogen form changes, but it is not assimilated (Dodds, 2002). These groups of bacteria develop mainly in oligotrophic environments (Ward, 1996), and well-aerated soils. Moreover, Rydin and Jeglum (2006) indicate that in very acidic conditions (pH <5) this process is severely hampered. In fact, nitrification, due to releasing H^+ ions, increases the acidity of soils (Weil and Brady, 2017).

Under anaerobic conditions, a microbial process called dissimilatory nitrate reduction to ammonium (DNRA) can reverse nitrification. This pathway consists of the dissimilatory transformation of NO_3^- into NH_4^+, as oppose to assimilatory that would mean the uptake of N by the microbes and thus its immobilization (Burgin and Hamilton, 2007). Heterotrophic organisms, using as electron donor organic carbon (fermentative DNRA), perform DNRA; and it can be performed as well by chemolithoautotrophic organisms which to oxidize sulphide, or other reduced substrates, use nitrate; both pathways are indicated in the following equations (Giblin et al., 2013):

Fermentative DNRA

$$CH_2O + ½NO_3^- + ½H_2O \rightarrow NH_4^+ + HCO_3^- \quad \text{(Equation 1-6)}$$

Autotrophic DNRA

$$HS^- + NO_3^- + H^+ + H_2O \rightarrow SO_4^{-2} + NH_4^+ \quad \text{(Equation 1-7)}$$

As an anaerobic process, it is important in wetland ecosystems where it could be responsible for a wide range of NO_3^- removal, e.g. up to 100% in mangroves or up to 60% in riparian wetlands (Burgin and Hamilton, 2007). However, in tropical forest soils that are not as anoxic as waterlogged ecosystems Silver et al. (2001) found that DNRA accounts for up to 75% of the total NO_3^- removal. On the other hand, so far it has not been reported in peatlands yet.

Denitrification can be explained as the process in which anaerobic bacteria reduce NO_3^- to N_2O or N_2 (Rydin and Jeglum, 2006). This is the anaerobic respiration of NO_3^- which is less effective than the aerobic one because O_2 is more oxidized than NO_3^- and produces more energy when reacting with organic matter (Dodds, 2002). This is a key process within the nitrogen cycle because it links the inorganic Nr form (NO_3^-) to the almost not available form of N_2 gas (Dodds, 2002), and it is carried out mostly by heterotrophs, although there are autotrophs as well within the denitrifying bacteria group (Weil and Brady, 2017). To do so there are four steps: first NO_3^- is reduced to NO_2^-, then to NO, next to N_2O, and finally to N_2 as represented in the following equations (Weil and Brady, 2017):

$$2NO_3^- \xrightarrow{-2O} 2NO_2^- \xrightarrow{-2O} 2NO\uparrow_{gas} \xrightarrow{-O} N_2O\uparrow_{gas} \xrightarrow{-O} N_2\uparrow_{gas} \quad \text{(Equation 1-8)}$$

This is a widespread process and the bacteria responsible for it are mostly present in large numbers. The ones that can perform all the steps are called classical denitrifiers, but many microbes cannot complete the pathway releasing NO or N_2O gases to the environment (Stein and Klotz, 2016). It is a paramount process with relation to the nitrogen loss of peatlands and forests, but also to the source of N_2O that is an important greenhouse gas. However, in

acid environments such as bogs denitrification occurs at very low rates, so that these areas may accumulate important quantities of immobile organic nitrogen (Etherington, 1983), they work as N sinks. On the contrary, recent studies have found that peatlands exposed to high rates of Nr deposition have also shown high rates of denitrification (Sgouridis et al., 2015).

Anammox stands for anaerobic ammonium oxidation and can be considered as an intermediate process between nitrification and denitrification. It is the transformation of NO_2^- and NH_4^+ to N_2, and ammonia-oxidizing archaea and ammonia-oxidizing bacteria are the two most important microbial groups involved in this process (Zhou et al., 2014), that can be represented as follows (Weil and Brady, 2017):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad \text{(Equation 1-9)}$$

This process is very beneficial from the environmental point of view because it removes the nitrogen (both ammonium and nitrite) without generating N_2O (Stein and Klotz, 2016), however, so far, it has not been reported in peatlands yet except for laboratory experiments using peat as substrate (Hu et al., 2011).

1.2 Nitrogen deposition

1.2.1 Nitrogen emission and deposition in the UK

Since the industrial revolution, the contribution of anthropogenic Nr to the biosphere has increased enormously because of three principal activities: agricultural intensification, fertilizer production and fossil fuel combustion (Gundale et al., 2011). The intensification of legumes crops (which are associated with N_2-fixers) combined with the addition of Nr to

crops using industrial fertilizers has become the main source of Nr to the biosphere (Billen et al., 2013). These fertilizers are obtained through the process known as Haber-Bosch and started to be produced at industrial scale at the beginning of the 20th century (Vojvodic et al., 2014). By 2008 the amount of ammonia produced by this method was about 100 Tg per year (Erisman et al., 2008), and just 3 years later it was 108 Tg N per year (Kandemir et al., 2013), continuing the increasing trend of fertilizer production since it started (Erisman et al., 2008). This process requires not only high temperatures (typically 500°C) and high pressures (typically 200 bar), but essentially a good catalyst (e.g. osmium, ruthenium; Kandemir et al., 2013). Under these conditions large amounts of ammonia can be achieved according to the following reaction (Vojvodic et al., 2014):

$$N_2 + 3H_2 \rightarrow 2NH_3 \qquad \text{(Equation 1-10)}$$

The other main anthropogenic input is energy (fossil fuel combustion) that creates Nr indirectly, this is that nitrogen is emitted to the air as a resultant product (NO) from either the oxidation of organic N or atmospheric N_2 in the fuel (Galloway et al., 2004). In fact, Elser (2011) claims that there is no part of the world free of anthropogenic Nr because it is awash with N. In addition, Elser (2011) highlights that a study found in an ice core from Greenland changes in the isotopic signature of NO_3 in agreement with an increase in the use of fertilizers and fossil fuels; and that other study found that an increase in Nr deposition in tropical rainforests has alleviated their N limitation and changed their isotopic composition.

In the UK, the emission levels of NOx have been reduced by more than two thirds (68%) between 1990 and 2014 (Fig. 1.4). The main source of nitrogen oxides (NOx) is combustion,

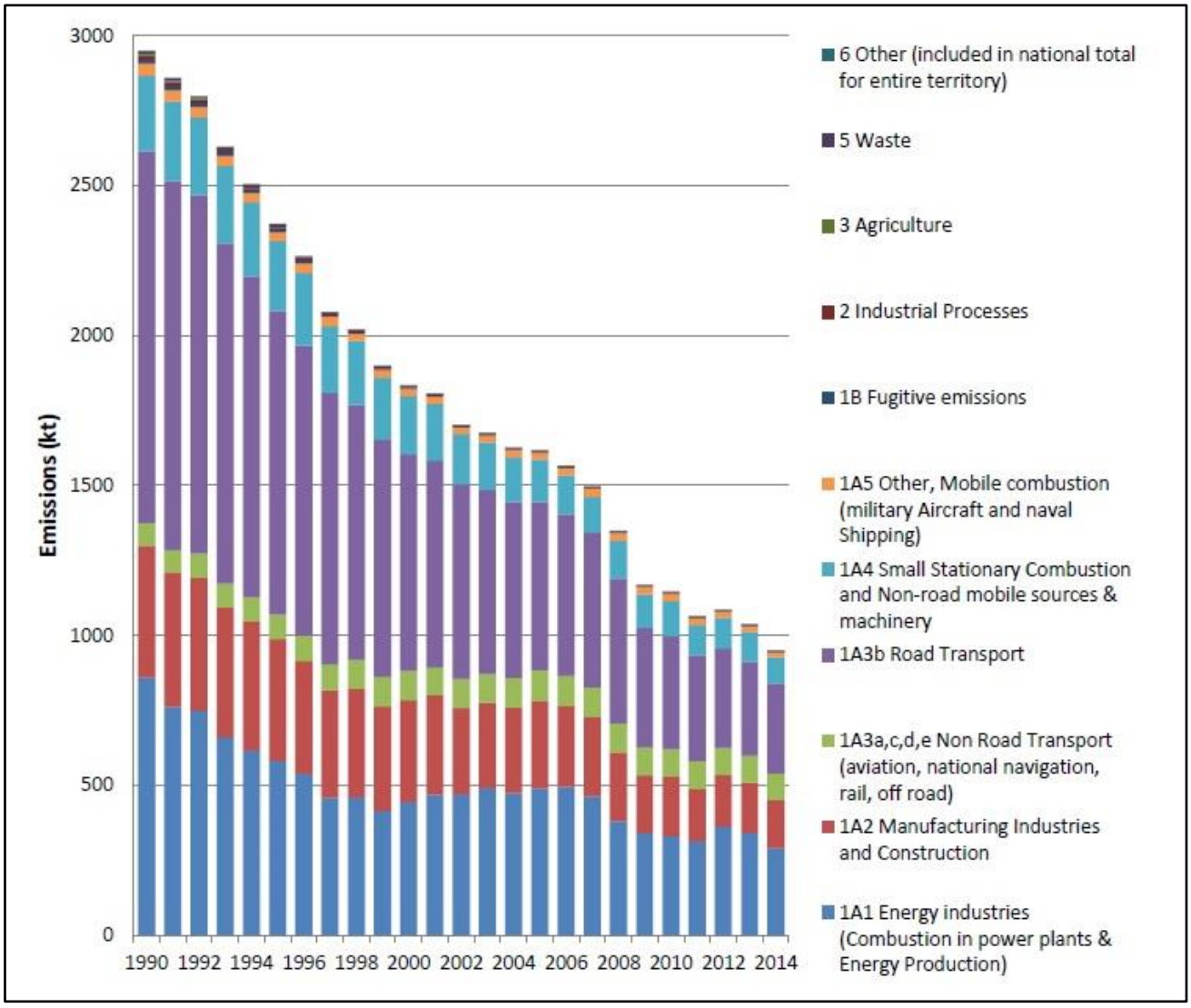


Figure 1.4. Total UK Emissions by Source Sectors of Oxides of Nitrogen (NOx as NO_2), 1990-2014. (Source: Wakeling et al., 2016)

and it can be observed in Figure 1.5 (red lines following the main roads) that the largest contribution is done by road transport (e.g. 33% in 2010), and the second most important one by energy industries (e.g. 25% in 2008). It is in the main cities and the main roads that connect them where the NOx emissions are concentrated, and considering the whole of Britain, the major emissions arise from England (Fig. 1.5). The projections for 2020 in the UK are to continue with the present diminution trend in order to reach an emission amount of NOx (as NO_2) of 728 kilotonnes, this is 222 kilotonnes less than in 2014 (Wakeling et al.,

2016), and by 2016 the emissions where 893 kilotonnes so closer to the 2020 target (Wakeling et al., 2018).

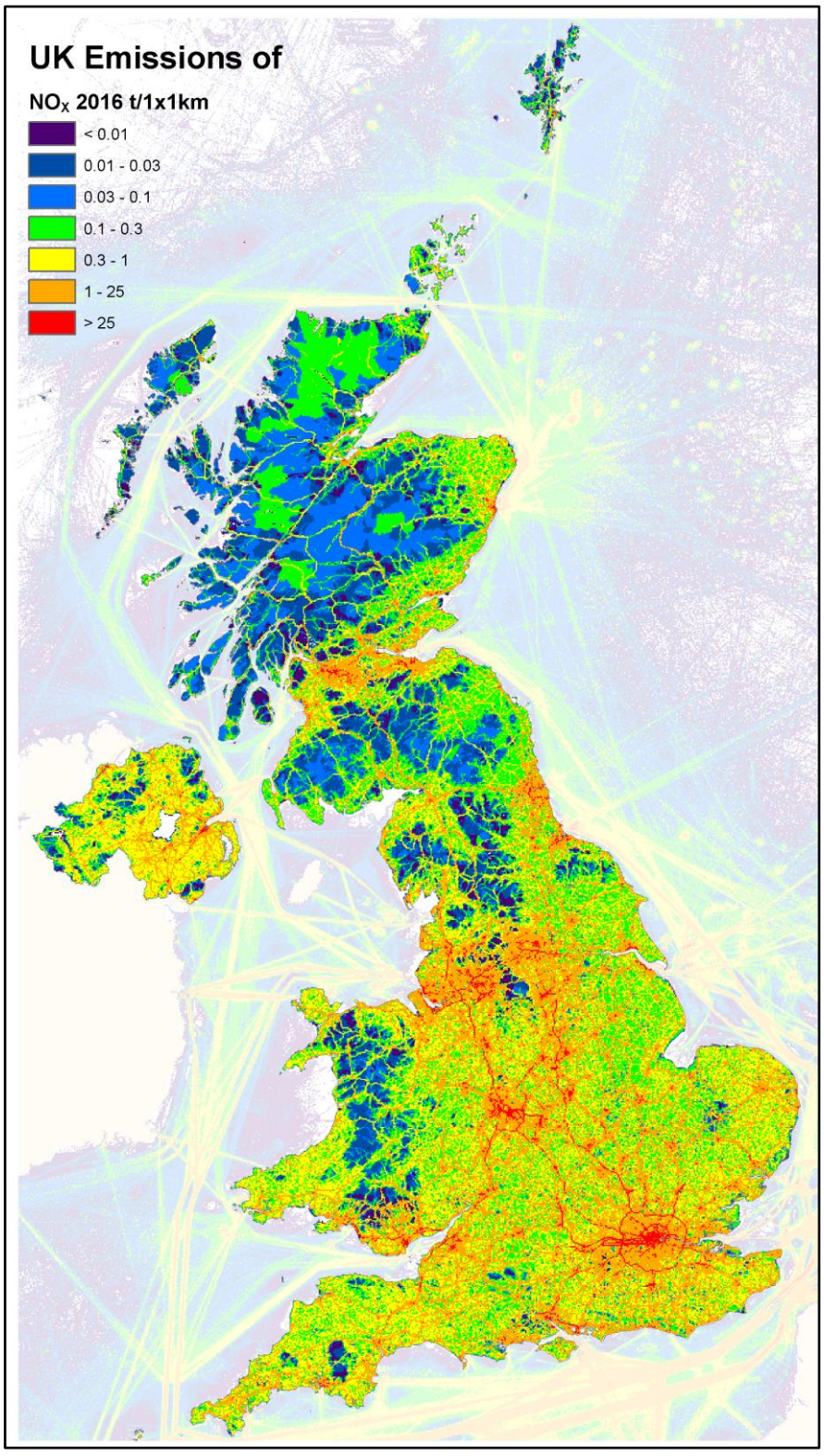


Figure 1.5. Map of NOx emissions in UK 2016 (expressed as tonnes NO_2 per km^2). (Source: National Atmospheric Emissions Inventory)

The pollution by ammonia is mainly a result of fertilizer volatilization and intensive animal farming, and the 90 % of ammonia emissions come from agriculture (Fig. 1.6; Burch, 2001; Wakeling et al., 2016). In the UK, air emissions of ammonia within the agricultural sector are dominated by cattle accounting for 47% in 2008, while the second and third major sources are livestock and synthetic fertilizer application that contributed for 30% and 11% of the UK total in 2007 (RoTAP, 2012). The levels of ammonia emissions in the UK, 281 kilotonnes for 2014, were below the NECD (EU National Emissions Ceiling Directive) ceiling of 282 kilotonnes (Wakeling et al., 2016), however by 2016 the emissions of ammonia were 10% above the lowest level reached in 2008 (Wakeling et al., 2018). Figure 1.7 shows the distribution of NH_3 in the UK. It can be observed high emissions in East Anglia which is due to pig farming and poultry; and in Northern Ireland, north of England, and south-west which is due to poultry, cattle and pigs (RoTAP, 2012).

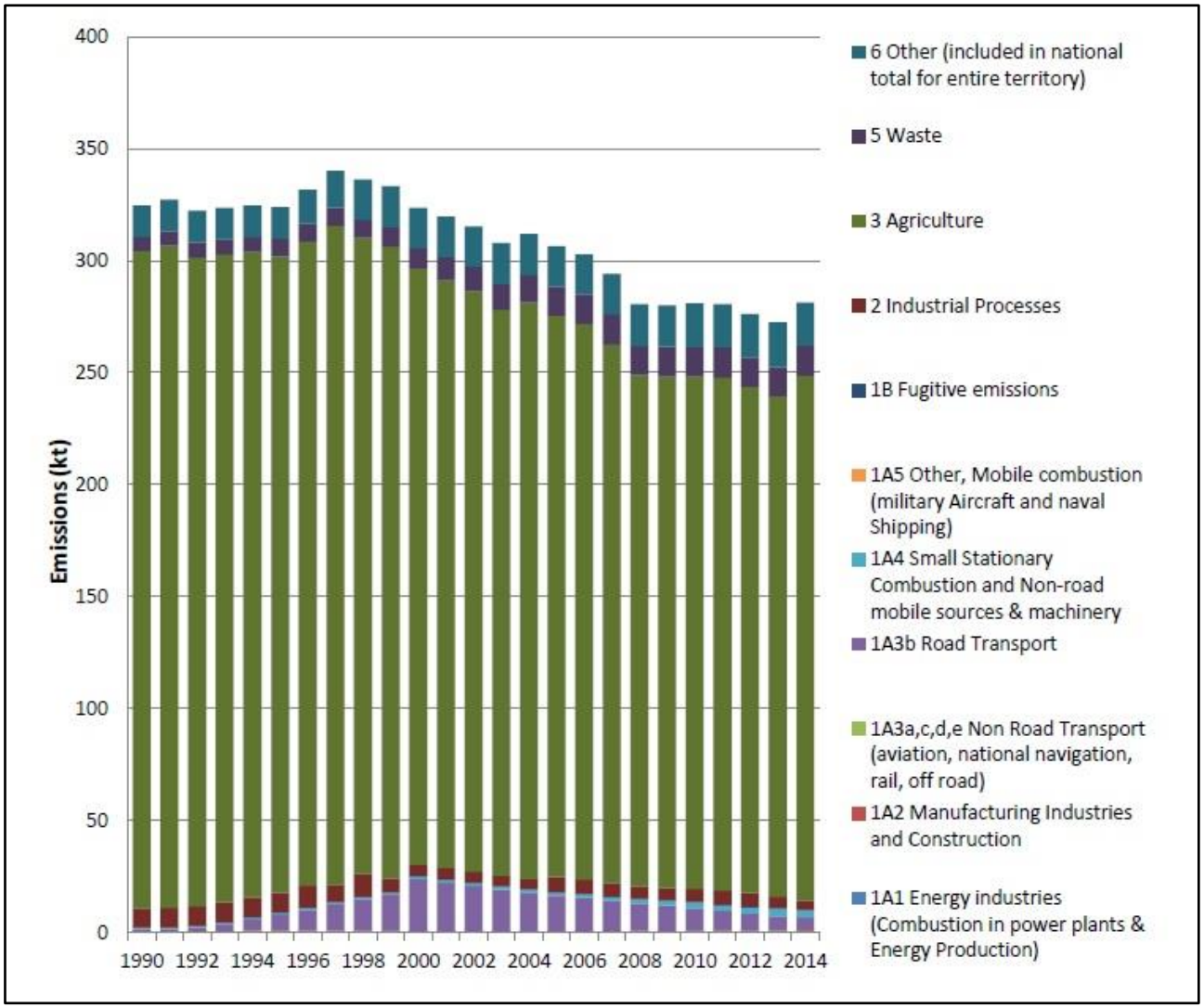


Figure 1.6. Total UK emissions by source sectors ammonia (NH_3), 1990-2014. (Source: Wakeling et al., 2016)

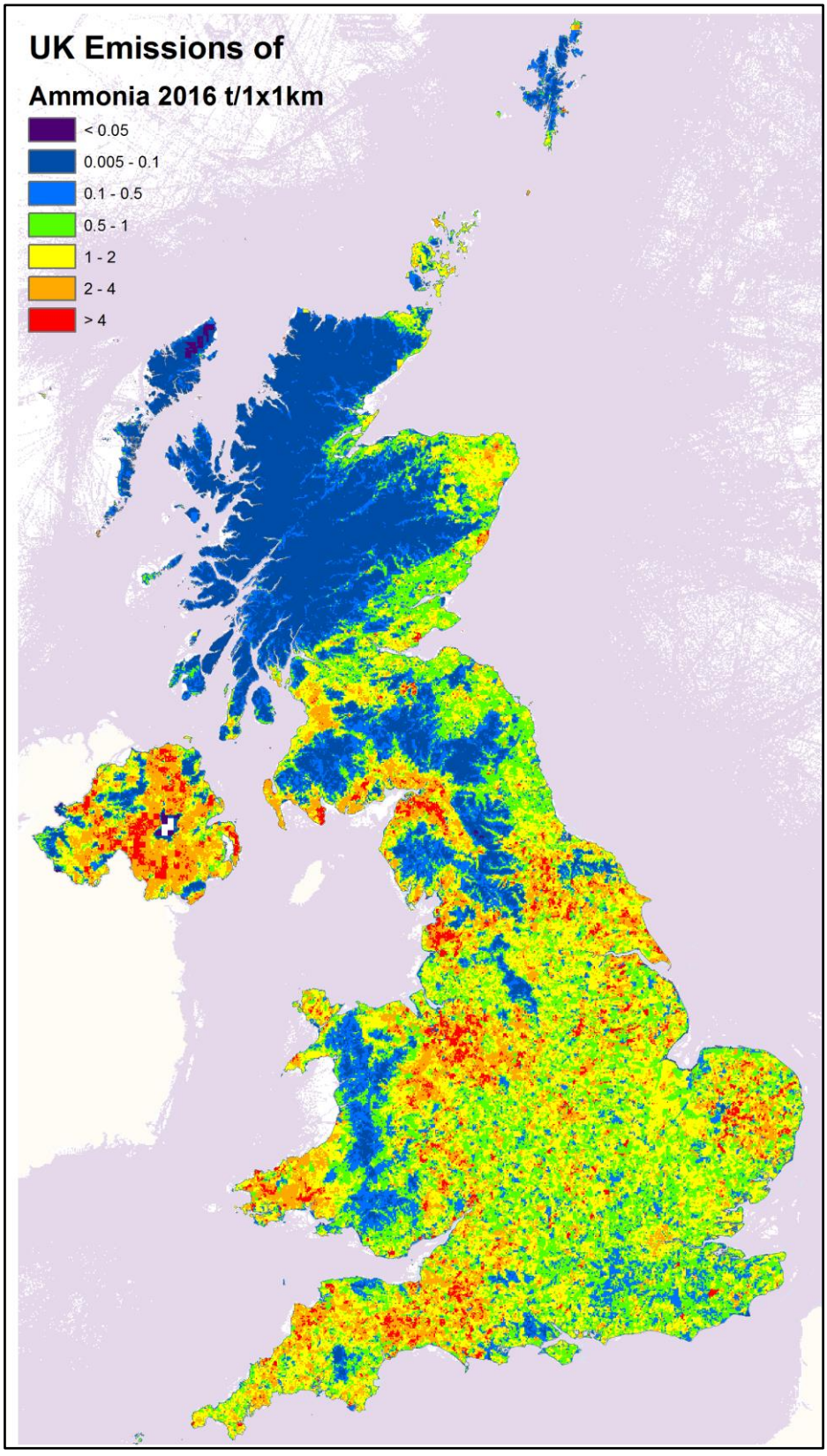


Figure 1.7. Mapped emissions of NH_3 in 2016 in UK expressed in t NH_3 per km^2. (Source: National Atmospheric Emissions Inventory)

These reductions in emissions have not been translated to equivalent declines in deposition (Stevens et al., 2016). For example, between 1986 and 2007 NOx emissions in UK fell by 50%, but nitrogen deposition fell by just 22% (Field et al., 2014). It has been suggested that this discrepancy is because chemical reactions are changing in the atmosphere going to a more rapid conversion of nitrogen dioxide to nitric acid and particulate nitrogen (Field et al., 2014).

Wet and dry deposition are the way by which NHx and NOx are deposited on land surface (Burch, 2001). Wet deposition involves the scavenging of nitrogen particles from the atmosphere by precipitation, while dry deposition means that particles are removed by gravitational settling, impaction, molecular diffusion and turbulent transport (Burch, 2001; Reay et al., 2008). In the UK the total reactive nitrogen rates (oxidised and reduced) have reached high values ranging from < 10 kg N ha^{-1} yr^{-1} in the north of Scotland to > 24 kg N ha^{-1} yr^{-1} in the north west of England and most of Wales (Fig. 1.8). Payne (2014) reported that during the decade to 2010 the mean nitrogen deposition value received by the British peatlands was 14.1 kg N ha^{-1} yr^{-1}, and that the predictions are not good even for the areas of less nitrogen deposition such as Forsinard Flows National Nature Reserve in northern Scotland where it is predicted to increase the deposition in 50 % by 2030 from 6.3 kg N ha^{-1} yr^{-1} to 9 kg N ha^{-1} yr^{-1}. In forests is more difficult to get a precise figure of atmospheric Nr deposition because of the "edge effect", i.e. throughfall Nr deposition is four times higher in the forest edge than in the interior (Wuyts et al., 2008).

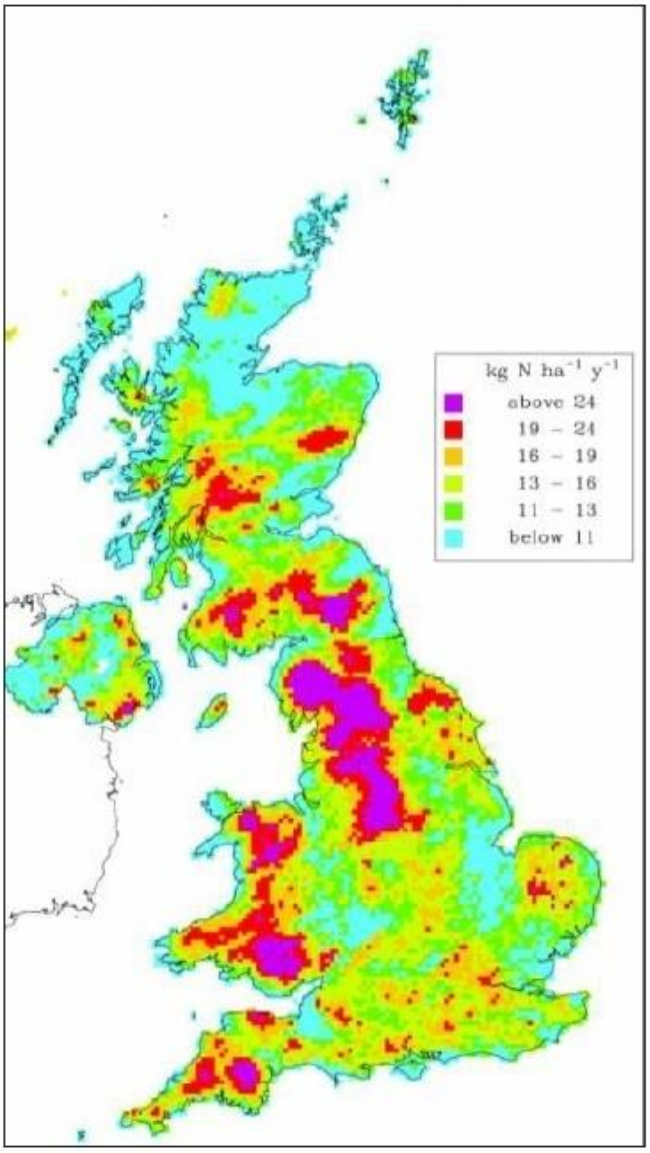


Figure 1.8. Total deposition of N over the UK in 2015. (Source: National Atmospheric Emissions Inventory)

1.2.2 Effects of increased Nr deposition in peatlands and forests

One of the most important factors that determine the composition of species in ecosystems is the availability of nutrients (Bobbink and Lamers, 2002). In forests and in most of the peatlands such as bogs, the limiting nutrient for plant growth is nitrogen (Urban and Eisenreich, 1988; Son, 2001).

Under the nutrient-limited and acidic conditions of bogs, which are 'engineered' by *Sphagnum* mosses mainly through the filtration of nutrients derived from atmospheric Nr deposition and BNF, they can outcompete vascular plants (Fritz et al., 2012). The maintenance of these conditions favourable for *Sphagnum* mosses allows the bog to develop and increase peat accumulation. However, an increase in Nr deposition rates, combined with higher temperatures, may transform bog ecosystems dominated by *Sphagnum* into others dominated by vascular plants, with very different functions and appearance (Manninen et al., 2011). The focus has been on the ecosystem level and particularly on species composition. The mechanisms of this transformation are very complex, but a sequence of three stages was defined (Berendese et al., 2001). First, if the Nr deposition is at low levels, the tight mat of *Sphagnum* mosses intercepts the Nr to reduce its availability to associated vascular plants (Millet et al., 2012), the mosses use the additional Nr to grow and there is no accumulation on tissues (Aerts et al., 1992). Second, if the Nr deposition increases (e.g. 12-18 kg ha^{-1} yr^{-1}) the growth can become P-limited (Limpens et al., 2004) and the excess of nitrogen is accumulated in the tissue as N-rich free amino acids and free N and at this levels other more nitrophilous *Sphagnum* species (e.g. *Sphagnum recurvum*) and vascular plants get advantage (Lamers et al., 2000). Finally, Bobbink and Lamers (2002) observed that higher rates of Nr inputs (>20 kg ha-1 yr-1) did not show increases in N concentration in *Sphagnum* mosses tissue, so N is leached into the peat soil where it is available for vascular plants. At this point, Berendese et al. (2001) suggest that vegetation community will shift and be colonized by vascular plants.

Chronic elevated Nr additions on temperate forests may cause changes in their biogeochemistry and structure. Four stages of increased Nr additions that lead to a final forest decline have been defined. The first one, an increase in Nr deposition would result in an increase in N uptake by the trees' canopy that would be used for growth and increasing

carbon sequestration (Aber et al., 1989; De Vries et al., 2006; Tipping et al., 2017). In the second place, under continuous high levels of Nr deposition canopy interception would fail and Nr would reach the ground, where after a while the vegetation community would change into the more nitrophilous ones, reducing biodiversity as a result (Kennedy, 2003; Dirnböck et al., 2014; Simkin et al., 2016). Next, when N is no longer a limiting element for trees, what would happen is a deficit in other nutrients leading to a nutrient imbalance that weakens the trees becoming thus more susceptible to pests and increasing water stress (Aber et al., 1989; Schulze, 1989). Following, when there is no uptake capacity by trees, nor soil store capacity, the result is nitrate "leaking out" of soil. This leakage would cause the acidification of soil and seepage waters that would increase Al toxicity for trees and stream waters (Van Breemen and Dijk, 1988; Gundersen et al., 2006). Finally, all previous effects would lead to a general forest decline due to the disruption of the forest structure (Van Breemen and Dijk, 1988; Aber et al., 1989; Schulze, 1989).

BNF is also affected by the increase of Nr deposition rates, and there is still little understanding of its mechanism (Rousk and Michelsen, 2016). Most of the most recent studies related to the influence of increased Nr deposition rates on BNF has been done in boreal forests with the ubiquitous feather mosses (e.g. Zackrisson et al., 2004; Leppänen et al., 2013; Rousk et al., 2014; Salemaa et al., 2019). In a study in a boreal forest in Sweden with feather mosses (*Pleurozium schreberi*) and a background of Nr deposition rates <0.5 kg N ha^{-1} yr^{-1}, Zackrisson et al. (2004) found almost total suppression on the BNF rates when applying a fertilization treatment of 4.25 kg N ha^{-1} yr^{-1}. Another study at the same location showed that after three years of N fertilization treatments at a rate of 25.5 kg N ha^{-1} yr^{-1} no N_2-fixers were found on moss shoots (*P. schreberi*; DeLuca et al., 2007). However, Gundale et al. (2013) found that in the case of the feather moss *P. schreberi* there was no response in the BNF rates after 16 years of different N fertilization treatments (12.5 and 50 kg N ha^{-1} yr^{-}

^{1}), whereas for *Hylocomium splendens* the BNF rates were reduced by 58% and 97% in the low and high treatments respectively (Fig. 1.9). In the case of temperate forests, BNF rates in forest soil decreased by 56% after the addition of a combination of nutrients (~1 g Mo ha^{-1}, 10.3 kg P ha^{-1}, and 7.7 kg N ha^{-1}) during the incubation (Perakis et al., 2017). Similarly, in temperate peatlands, the effects of high rates of Nr deposition are not clear. The results of a mesocosm experiment with *Sphagnum* mosses from peatlands under high and low background levels of Nr deposition showed that Nr fertilization treatments reduced BNF rates in mosses from both areas (Kox et al., 2016). On the other hand, van den Elzen et al. (2018) found that after 11 years of two Nr fertilization treatments (8 and 32 kg N ha^{-1} yr^{-1}) BNF rates of *Sphagnum* mosses remained unaffected. So, it is not clear how increased Nr deposition affects BNF.

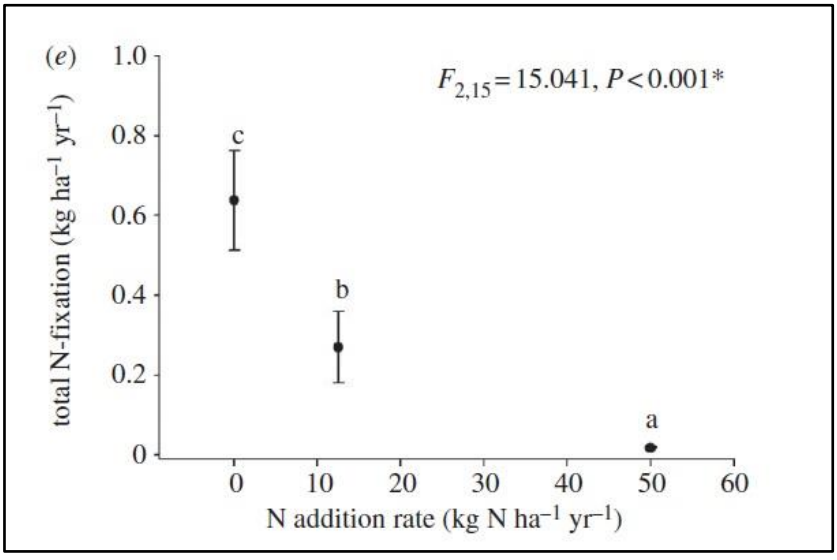


Figure 1.9. Total BNF rates per unit area in response to 16 years of experimental Nr addition treatments (0, 12.5 and 50 kg ha^{-1} yr^{-1}). (Source: Gundale et al., 2013)

1.2.3 The Nr 'critical load' for peatlands and temperate forests in the UK

The concept 'critical load' has been used since the 1980s (Jefferies and Maron, 1997; Bragazza et al., 2004). Nilsson (1988, p. 85) defined critical load of a pollutant as 'the highest load that will not cause chemical changes leading to long-term harmful effects on most sensitive ecological systems.' Gunnarsson and Rydin (2000, p. 528) used the definition applied to nitrogen by Greenfelt and Thörnelöf (1992) who defined critical load as 'a quantitative estimate of an exposure to a deposition of N as NHx and/or NOy below which empirical detectable changes in ecosystem structure and function do not occur according to present knowledge.' Despite the efforts to define the term, it is very difficult to calculate the critical load value for a particular pollutant (Burch, 2001). In fact, if a value is established for a pollutant in a specific place it can vary between places and populations (Jefferies and Maron, 1997).

For ombrotrophic *Sphagnum* plants, which are usually found in mires or bogs, it has been defined the critical load as 'the amount of bulk deposition above which *Sphagnum* plants experience nutrient imbalance to such an extent to greatly decrease the absorption of exogenous N' (Bragazza et al., 2004, p. 615). Numerous studies have been focused on the effects of elevated levels of atmospheric Nr deposition on ombrotrophic *Sphagnum* plants (Gunnarsson and Rydin, 2000; Bobbink and Lamers, 2002; Bragazza et al., 2004) and from the results it has been calculated that the critical load value is approximately 10 kg N ha^{-1} yr^{-1}, above which *Sphagnum* plants shift from being N-limited to being P + K co-limited (Bragazza et al., 2004). As an environmental pollutant, later attempts to establish a critical load for Nr deposition were done by Payne et al. (2013) and they reported that it was possible to identify changes at Nr deposition rates of 7 kg N ha^{-1} yr^{-1}. This value is within the range of the empirical critical loads of Nr that were defined in 2010 for the mire, bog and fen habitats in the UK between 5 and 10 kg N ha^{-1} yr^{-1} (Hall et al., 2011).

To establish a critical load of Nr for forest habitats is more complex. The sensitivity of the plants to the addition of Nr is inverse to the availability of N, i.e. the more N that is normally available the less sensitive to Nr addition, and it is also related to the type of plants (Kuylenstierna et al., 1998). No evidence of changes in tree composition in forests due to high rates of atmospheric Nr deposition has been found so far, however, it has been related with changes in the understorey vegetation and forest decline (Aber et al., 1998; Becker et al., 2017). This may be because it would take from decades to hundreds of years to see changes in tree composition in forests, in contrast to the more rapid changes in herbaceous species (Jefferies and Maron, 1997). The response of the understorey vegetation community to Nr deposition depends on four different aspects. First, the Nr deposition forms could vary between sites and thus originate different responses. Second, different overstorey composition between sites would lead to a different filtration of the Nr compounds and thus trigger a different response of the understorey vegetation species as well as of the soil biogeochemistry. Third, differences between regions in biodiversity would lead to different effects of Nr deposition. And fourth, variations at the landscape scale, e.g. distance to the forest edge, would influence the response to the Nr deposition (Perring et al., 2018). Because of this complexity, the empirical critical load for temperate forests in the UK has been defined considering the different forest habitats: coniferous woodland between 5 and 15 kg N ha^{-1} yr^{-1}; broadleaved woodland between 10 and 20 kg N ha^{-1} yr^{-1}; all forests (ground flora) between 10 and 15 kg N ha^{-1} yr^{-1} (Bobbink and Hettelingh, 2010).

1.3 Biological nitrogen nixation

1.3.1 Dinitrogen fixation

The first paper related to BNF was published in 1888 and since then our knowledge about it has increased enormously (Galloway et al., 2004). The main natural input of reactive N to the biosphere is through BNF by a group of microorganisms called diazotrophs (Newton, 2007). In order to incorporate N into organic material, it must be in the ammonium (NH_4^+) form, which is one of the products of BNF. Smith and Gallon (1993) exemplified the BNF equation as:

$$N_2 + 10H^+ + 8e^- \rightarrow 2NH_4^+ + H_2 \qquad \text{(Equation 1-11)}$$

Which is an exergonic reaction, this is it gives out energy although organisms have to provide energy to start the reaction (16 molecules of adenosine triphosphate -ATP- per molecule of dinitrogen reduced), and it takes place in an aqueous environment where an input of electrons is needed because hydrogen is replaced by protons. The organisms that can fix nitrogen, diazotrophs, are divided into many categories and subcategories, and it makes very complicated to have clear in a first instance the group of organisms that different papers referred to because they use different terms. A basic and understandable division, but not exhaustive, was done by Sprent and Sprent (1990) who divided diazotrophs into free-living and symbiotic; then free-living are divided into archaeabacteria (methanotrophs) and eubacteria; eubacteria are divided into heterotrophs (anaerobes, facultative anaerobes/microaerobes, aerobes) and autotrophs (chemotrophic bacteria, photosynthetic bacteria, and cyanobacteria -blue-green algae-: unicells, filamentous forms, and heterocystous forms); moreover, symbiotic are divided into rhizobiaceae, actinomycetales

and also a group called cyanobacteria. This last group is one typically associated with bryophytes that could be found in peatlands and forests, while free-living ones are normally present in soils (including peat). The cyanobacteria can be endophytic, i.e. that lives within the plant, or epiphytic, i.e. that lives on the surface of the plant (Turetsky, 2003).

Within these organisms the N_2-fixing enzyme is nitrogenase (Sprent, 1979; Postgate 1982). Until 1980 only one type of enzyme nitrogenase was known, but today three genetically different enzymes of nitrogenase are recognized: the canonical molybdenum-based nitrogenase (Mo-nitrogenase), the vanadium-based nitrogenase (V-nitrogenase) and the one based on iron alone (the other two also contain iron) that is called more often 'alternative' nitrogenase (Smith and Gallon 1993; Newton, 2007). It is known that Mo-nitrogenase is present in all diazotrophs, but only some have V-nitrogenase or Fe-only nitrogenase, and the methods to measure the fixation rates did not differentiate between the nitrogenases so it was not possible a full understanding of BNF (e.g. metal limitations). Recently, Zhang et al. (2016) have presented a new method that distinguishes the three nitrogenases so it is going to be possible to go further in the interactions between trace metals and N cycle and our understanding of the controls of BNF.

1.3.2 Biological nitrogen fixation in mosses and soil

Sphagnum mosses seem to be excellent hosts for microorganisms responsible for BNF (Opelt et al., 2007). Initially, it was considered that in ombrotrophic bogs (very acidic environments) it was not possible to find N_2-fixers that were not adapted to acidic conditions as, for example, blue-green algae. However, Granhall and Hofsten (1976) reported that intracellular organisms have been found in *Sphagnum* mosses such as bacteria, green algae, fungi, and blue-green algae. In particular, they were found in the hyaline cells, that are full

of water, and constitute a microhabitat for them. The ability of the *Sphagnum* to acidify the exterior through cation exchange generates an increase of pH inside the plant that facilitates the microorganisms to stay in such acidic exterior environments (Granhall and Hofsten, 1976). Turetsky (2003) affirms that there is no concurrence with respect to the BNF rates between *Sphagnum* and cyanobacteria associations. This is because of the results of contradictory studies such as the one of Schwintzer (1983) that reported no N_2-fixing cyanobacteria in association with *Sphagnum* in a minerotrophic peatland. Or the one of Urban and Eisenreich (1988) that found in a *Sphagnum*-dominated forested peatland a rate of 0.5-0.7 kg N ha^{-1} yr^{-1}.

In temperate forests, it is common to find feather mosses such as *Hypnum cupressiforme* in more acidic bark and rocks or *Eurhynchium striatum* in more base-rich soils (Atherton et al., 2010). The feather moss associated diazotrophs are usually cyanobacteria and they are found on the moss leaves surface (epiphytically; Rousk et al., 2018). Thus, the N_2 fixed would be quickly available for other individuals such as trees through the bark (Tukey, 1952), apart from the host (Rousk et al., 2018). Epiphytic mosses play a key role as a source of N for their hosts (Lindo and Whiteley, 2011; Jean et al., 2012), and therefore they should be considered while studying BNF in forests.

But not all N_2-fixers are in association with mosses, they can be in soil (free-living). It has been found aerobic bacteria in the oxic upper layer of peat, and anaerobic bacteria in the waterlogged and then anoxic layer of peat (Chapman and Hemond, 1982). Chapman and Hemond (1982) reported a list of studies in the seventies that were focused on the BNF rate in peat soil ranging from 0.3 to 60 kg N ha^{-1} yr^{-1}, so very different results that made clear the existence of abiotic factors affecting BNF and the need of further study. In forested ecosystems, non-symbiotic BNF is an important source of N (Cleveland et al., 1999) that could reach inputs up to 50 Kg N ha^{-1} yr^{-1} (Bormann et al., 1993; Son, 2001). However, in

most of the non-symbiotic BNF measurements done in temperate forests with the acetylene reduction method the rates varied between <0.01 and 12 kg N ha^{-1} yr^{-1} (Son, 2001), remaining unaccounted up to 38 kg N ha^{-1} yr^{-1}, which could be because of an underestimation due to the method used.

The improvement of new methods in the seventies and eighties to measure BNF allowed researches to develop new projects focused not only on BNF surveys across the world but also focused on the relationships of BNF and physical and chemical factors as well as peatland ecology. These investigations led to a better understanding of the controls of BNF.

1.3.3 Controls of biological nitrogen fixation

Since the seventies, it was clear that factors were influencing the BNF rates such as physical factors, e.g. light and temperature, or chemical factors, e.g. pH, phosphorous, and potassium (Chapman and Hedmon, 1982). An example of a broad study on BNF rates and the factors that controlled the process was reported by Waughman and Bellamy (1980) and consisted of a survey of the BNF rates of peat from eight different countries (Scotland, England, Ireland, Germany, Norway, Malaysia, Italy and Canada), and a deeper investigation in three different peatlands in southern Germany in order to gain understanding in the relationship between BNF and its ecological controls. This study concluded that the highest rates of BNF were in fens and the lowest in bogs; and that three factors had a significant correlation with BNF: Ca, K and pH.

Many environmental factors influence BNF activity such as temperature, moisture, oxygen, light, nutrients, or pH (Chapin and Bledsoe, 1992; Belnap, 2001). The presence of oxygen affects BNF as nitrogenase enzyme works under anoxic conditions (Posgate, 1982). In the laboratory, Fay (1992) found that BNF of cyanobacteria increased as dissolved oxygen, in

the culture medium, decreased. And Warren et al. (2017) found that the presence of oxygen in peat incubations decreased BNF by 90%. Other studies have focused on what seems to be the key factors controlling BNF rates such as moisture, temperature and light (Sorensen and Michelsen, 2011; Sorensen et al., 2012; Caputa et al., 2013; Rousk et al., 2015). About temperature, it has been reported that between 20 and 30 ºC is the optimum in which nitrogenase activity occurs (Caputa et al., 2013; Rousk et al., 2015), reducing the BNF rates if the temperature is too low or too high. Another important element that controls BNF is light, particularly in autotrophs, because heterotrophic diazotrophs take the energy from organic matter (Sorensen et al., 2012; Rousk et al., 2015). Less light due to an increase in vascular plants and more organic matter available can lead to decreased abundance of mosses, and thus to lower BNF rates (Deslippe et al., 2005; Sorensen and Michelsen, 2011; Sorensen et al., 2012). Concerning the global distribution of BNF, Houlton et al. (2008) found that symbiotic N_2-fixing organisms have an advantage in P-limited tropical forests and savannas and that the distribution of BNF is constrained by temperature at high latitudes. However, warmer temperatures combined with less frequent precipitation have been found to reduce the content of feather moss moisture and BNF rates in boreal forests of Sweden (Sorensen et al., 2006; Gundale et al., 2012). Although in subarctic regions the highest rates were found during the summer period or growing season (Stewart et al., 2011a), some experiments suggested that warmer temperatures can interact with litter releasing nutrients and increasing the BNF rates because they were P-limited (Liengen, 1999; Lett and Michelsen, 2014). All the studies mentioned above related to BNF controls are located mainly in boreal and arctic ecosystems, so further research is needed in temperate regions.

Another key factor that influences BNF is nutrient content. High amounts of nitrogen deposition reduce BNF rates (DeLuca et al., 2007; Vitousek et al., 2013). Zackrisson et al. (2004) found that after the addition of 4.5 kg ha^{-1} yr^{-1} of Nr in feather mosses of a boreal

forest BNF was suppressed. Similarly, Ackermann et al. (2012) reported that in feather mosses of a boreal forest above an Nr deposition of 3 kg N ha^{-1} yr^{-1} BNF is inhibited. In contrast, in the same area, Rousk et al. (2014) reported that the threshold above which BNF is inhibited could be 10 kg N ha^{-1} yr^{-1}. So, there is not a unique threshold of Nr deposition above which BNF activity is inhibited in boreal ecosystems. Long-term Nr additions will lead to a reduction of the moss surface and an increase of the dominance of vascular plants (Berendse et al., 2001; Wiedermann et al., 2007; Bragazza et al., 2012; Berg et al., 2013; Rousk and Michelsen, 2016). An increase in atmospheric Nr input is generating a shift in peatbog plants from being N limited to being P limited (Bragazza et al., 2004; Zackrisson et al., 2004; Sorensen et al., 2012). However, experiments with P fertilization in boreal ecosystems have had different results, reporting no effects on BNF (e.g. Zackrisson et al., 2009) in some cases, as well as a slight increase in BNF rates (e.g. Zackrisson et al., 2004; Sorensen and Michelsen, 2011) in others. P is an essential element in BNF, and it is not limited in soils in Britain, so it is needed to clarify to what extent in nutrient-poor peatlands that are not N limited (due to high Nr deposition) any more, become P limited or not.

Moreover, the presence of salts, which is linked to electrical conductivity (EC), also affects BNF. In fact, it has been reported for legumes that BNF rates decreased as EC increased (Bruning et al., 2015); and that BNF is even inhibited under salt-stress (Bolaños et al., 2006). However, a study carried out in a marsh ecosystem found that BNF decreased as salinity increased only for one of the two species of the study, with no conclusive results (Černá et al., 2009). For peatland ecosystems, there are no concluding studies looking at this factor so far.

Landscape topography is an important factor influencing BNF. Stewart et al. (2011b) reported that there were substantial differences between the hummock-hollow complexes in biological soil crusts of the low arctic tundra (Canada) regarding BNF rates. In particular,

they found that the areas of higher BNF rates were the lower part of the hummocks and the bottom of the hollows, and that it could be linked to higher availability of nutrients and moisture. Therefore, studying BNF rates in hollows and hummocks of *Sphagnum* mosses in peatlands would contribute to a better understanding of the effects of the landscape topography on BNF.

Recent studies have been focused on the interrelationships of the different N_2-fixing bacteria (heterotroph, phototroph, and methanotroph) in the moss vegetation, because all take part in the fixation process facilitating Nr to the plant. Although there is one study that did not find correlation (Leppänen et al., 2015) many others found that the oxidation of methane was linked to the BNF rate as the main energy source (Fig. 1.10), and it favoured higher rates of BNF (Larmola et al., 2014; Ho and Bodelier, 2015; Knorr et al., 2015). Likewise, it was reported that in addition to physical factors (e.g. temperature, light, moisture) BNF was governed by the depth of the water table, methane availability in the pore water, and concentrations of molybdenum (Mo), iron (Fe), or phosphorus (P) (Larmola et al., 2014). The rationale of the latter is that N_2 is fixed by the metalloenzyme nitrogenase which most common form is Mo-nitrogenase and contains molybdenum and iron, however, due to the fact that some other N_2-fixers possess V-nitrogenase and/or Fe-only nitrogenase, other authors (e.g. Marks et al., 2015; Zhang et al., 2016) suggest that vanadium is another metal that may influence BNF. Thus, studying these elements is essential to know their influence on BNF.

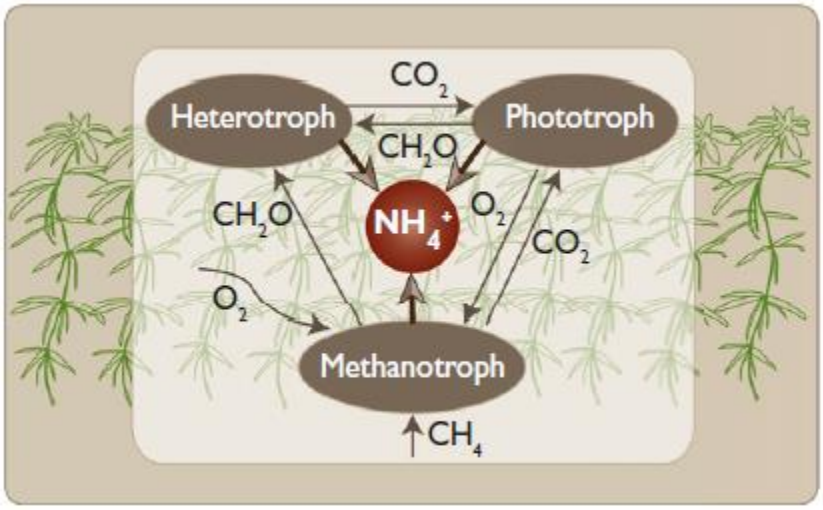


Figure 1.10. Interactions between the different N_2-fixing bacteria (methanotrophs, heterotrophs and phototrophs) in *Sphagnum*-dominated peatlands. (Source: Larmola et al., 2014)

1.3.4 Measurement of biological nitrogen fixation

There are two commonly used methods to measure BNF in peatlands and forests. One is the direct $^{15}N_2$ assimilation method in which samples that could be tissue, soil, or water, are incubated with $^{15}N_2$ to measure the increase in content of ^{15}N through time (Bellenger et al., 2014). Although it is a direct method, problems have been encountered while using it such as dilution problems of $^{15}N_2$ in water samples (Großkopf et al., 2012) or oxygen decrease in the incubation chamber due to long term incubations (Myrold et al., 1999).

The other and most common and widely used method to measure BNF is the acetylene reduction assay (ARA; Table 1.3). This is because it is cheap, easy to perform, and fast. It is based on that the nitrogenase enzyme preference of reducing C_2H_2 to C_2H_4 in the presence of the former, rather than N_2 to NH_3, even though the same requirements of reductant and ATP (Bergersen, 1970). Therefore, it is not direct, and it is necessary a conversion factor

that theoretically comes from the stoichiometry of the equation 1.11 and the following (Bellenger et al., 2014):

$$C_2H_2 + 2\,H^+ + 2e^- \rightarrow C_2H_4 + 2H_2 \qquad \text{(Equation 1-12)}$$

For some authors, the reduction of N_2 to ammonium requires 6 electrons (Bergersen, 1970) giving a theoretical conversion factor of 3 moles of C_2H_4 produced per mole of N_2 fixed, and for others it requires 8 (Staal et al., 2001) and the conversion factor then is 4:1. These theoretical conversion factors are close to the results of field and laboratory experiments that Hardy et al. (1968) did by using the direct $^{15}N_2$ to calibrate the indirect ARA giving a conversion factor between 3 and 4.5.

Table 1.3 shows that the ARA method is widely used to measure BNF in peatlands and forests, but also shows how variable is the conversion factor. In fact, it can be observed that the range goes from 8.5 to 0.25 which is a difference of one order of magnitude. In addition, since the early stages of the ARA method, authors encouraged to determine the conversion factor for each soil (Nohrstedt, 1983) or even condition of the samples (Bergersen, 1970). In other cases, the ARA failed to measure BNF activity in the presence of methane-utilizing bacteria (de Bont and Mulder, 1976), which is abundant in peatlands (Larmola et al., 2014) and temperate forest soils (Kolb et al., 2005). As a result, it is important to test how ARA performs in order to be sure that it is a robust and accurate method to measure BNF in peatlands and temperate forests.

Table 1.3. BNF rates and the method used in different peatland and forest ecosystems. ARA: Acetylene reduction assay method. $^{15}N_2$: direct assilimation method. NA: not applicable.

Location	Ecosystem	Main BNF method applied	Conversion factor (C_2H_4:N_2)	Estimated rate (Kg N ha^{-1} yr^{-1})	Incubated substrate	Nr deposition (Kg N ha^{-1} yr^{-1})	Reference
Pennine, UK	Bog	ARA		32	Peat	~30 (Curtis et al., 2005)	Martin and Holding, 1978
South German peatlands	Fen, poor fen, and bog	ARA	3:1	21, 5.3, 0.7	Peat	~12	Waughman and Bellamy, 1980
Thoreau's bog Massachusetts, USA	Bog	ARA	3.5:1	10	Peat and *Sphagnum* spp.	7	Chapman and Hemond, 1982
Petersham, Massachussets, USA	Open peatland	ARA	4:1	0, 0.4	*Sphagnum* spp., peat	~6.5	Schwintzer, 1983
Minnesota, USA	Forested bog	ARA	4:1	0.5-0.7	Peat	10.4	Urban and Eisenreich, 1988
Northern Sweden, Norway, and Finland	Boreal forest	ARA	3:1	1.5 – 2	*Pleurozium schreberi*	<2 (Leppännen et al., 2013)	DeLuca et al., 2002
South-east Manitoba	Boreal forest	ARA	3:1	1.93, 0.23	*Sphagnum capillifolium* and *Pleurozium schreberi*	<5.1 (Köchy and Wilson, 2001)	Markham, 2009
New Zealand	Forest	ARA	0.25:1	0.7-10	Bryophytes	<2	Menge and Hedin, 2009

Northwest Territories, Canada	Wet meadow	ARA	0.85:1	20.5	*Sphagnum* spp.	<1	Stewart et al., 2011a
Bothnian Bay, Finland	Meadows, fens, fen-bogs	$^{15}N_2$	NA	1-29	*Sphagnum* spp.	3	Lamola et al., 2014
Boreal Alberta, Canada	Bogs	ARA	0.32:1	25.8	*Sphagnum* spp.	1	Vile et al., 2014
Southern Patagonia, Chile	Bog	ARA	0.4-1.8:1	70-87 ($^{15}N_2$ method; 111 days growing season)	Peat	0.5	Knorr et al., 2015
Tierra de Fuego, Argentina; The Netherlands	Bogs	ARA	Not applied due to high variability	1160.49; 166.02 nmol C_2H_4 $g^{-1}DW\ d^{-1}$	*Sphagnum magellanicum*	0.5 >25	Kox et al., 2016
Copenhagen, Denmark	Bog	ARA	3:1	10.32	*Sphagnum* spp., *Pleurozium schreberi, Hypnum cupressiforme*	15	Rousk et al., 2018
Whim Bog, Scotland	Bog	$^{15}N_2$	NA	3-5	*Sphagnum* spp.	8	van den Elzen et al., 2018
North-central New Hampshire, USA	Hardwood forest	ARA	8.5:1	0.1-2	Wood litter (forest floor)	6.1 (Bowden, 1991)	Roskoski, 1980
Central Sweden	Coniferous and deciduous forests	ARA	1.61-5.61:1	0.4-1.4 kg N per growing season	Top forest soil (0-6 cm)	2-6	Nohrstedt, 1985
Ontario, Canada	Mature unmanaged forest stands of hardwood, jack	ARA	3:1	0.4, 0.06, 0.25	Forest organic soil (top 5 cm)	4.7	Hendrickson, 1990

	pine, and mixed wood						
Eastern Austria	Deciduous forest	ARA	5.4:1	0.002-0.008	Top forest soil	14 (Puxbaum and Gregori, 1998)	Zechmeister-Boltenstern and Kinzel, 1990
North-east Bavaria, Germany	Hardwood and coniferous forest	ARA	8:1	0.2	Upper organic soil layer	18-22 (Butterbach-Bahl et al., 1997)	Limmer and Drake, 1996
Maine, USA	Coniferous forest	ARA	4:1	0.06	Forest floor (top organic soil) and mineral soil (15 cm)	3-4.5	Barkmann and Schwintzer, 1998
British Columbia, Canada	Lodgepole pine	ARA	3:1	0.3-0.6	Woody debris and top organic soil (0-10 cm)	6.2	Wei and Kimmins, 1998
Duke Forest FACE Site, Orange County, North Carolina, USA	Experimental centre: 30 m diameter plots of loblolly pine	ARA		2.4-5.16 6	Forest floor and top soil (0-10 cm)	14 (Drake et al., 2011)	Hofmockel and Schlesinger, 2007
Chiloé Island, Chile	Temperate rain forest	ARA	3:1	1.3	Forest soil	<1	Pérez et al., 2010
Cape Canaveral, Florida, USA	Experimental centre: 9.42 m2 plots 2.5 m high of scrub oak palmetto.	$^{15}N_2$	NA	1-9	*Galactia elliottii*	~5 (Baril and Lapointe, 2005)	Hungate et al., 2014
Henan Province, China	Tropical forest Temperate forest	ARA	2.97:1 2.56:1	0.0134 0.0257	Forest soil	19.6	Zheng et al., 2019

1.4 Analytical techniques

1.4.1 Chromatography

Chromatography is a method by which the different components of a mixture can be separated and identified. There are different types depending on the techniques, materials, and equipment used, but all are based on the same principle. The different components of a substance are separated at a different migration rate across a stationary phase, while influenced by a mobile phase (Fifield and Haines, 2000; Ismail and Nielsen, 2010). In this thesis, we used two different chromatographic techniques: gas chromatography and ion chromatography.

- Gas chromatography. This is a technique, also consider as "column chromatography", in which the gas sample (or a liquid with a low boiling point) is injected into a column. The sample is transported by the flow of inert gas (in this case Helium), the mobile phase, through the length of the tube into the stationary phase that could be either an immobilized liquid or an inert solid packed in the column (Fifield and Haines, 2000; Ismail and Nielsen, 2010). The separation of the components of the gas mixture is achieved on the basis of various properties such as molecular size, polarity, and boiling point (Ismail and Nielsen, 2010). These components pass through a detector that determines the compound (time) and its amount (intensity of the signal). There are different types of detectors. Two different instruments were used based on the characteristics of the analyte and the compounds to be measured.
    - For gas samples (looking mainly at ethylene, acetylene, and ethane) a gas chromatograph equipped with a flame ionization detector (FID) was used due to its high sensitivity. The combustion of the sample generates different ionic

species that passing between two electrodes, in contact with the flame, generate a specific amount of current that identifies the compound present (Fifield and Haines, 2000).

  - For solid samples (moss and peat; looking at nitrogen, carbon, and sulphur), it was used an organic elemental analyser equipped with a thermal conductivity detector (TCD). It has not a very high sensitivity, but a wide response and it is the most adecuate for this kind of samples. The detector is based on the difference of the thermal conductivity in the mobile phase (Fifield and Haines, 2000).

- Ion chromatography. An ion chromatograph was used for looking at anions such as PO_4^{3-}, NO_3^-, SO_4^{2-} due to its high sensitivity, low amount of sample needed and simplicity of the operating system. In this case, the principle of separation is that between the mobile phase and the stationary phase there is a selective exchange of ions. Cations and anions have a varying affinity to the stationary phase so that they separate at different times and they can be identified in sequence. There is a system through which an eluent flows at low pressure (carrier), the sample is injected and transported to the column (stationary phase). As the sample reaches the column, the different components will move through the column at different speeds, leaving the column at a certain time they will go through the detector (based on conductivity) giving a specific signal (Fifield and Haines, 2000). This way, the anions and cations can be identified (time) as well as their amount (signal).

1.4.2 Mass spectrometry

Mass spectrometry is an important analytical method based on the different mass/charge ratios of the ions provided by a source (Perez-Arantegui and Laborda, 2018). Two main mass spectrometric techniques were used in this thesis: inductively coupled plasma – mass spectrometry (ICP-MS), and isotope ratio – mass spectrometry (IRMS).

- Inductively coupled plasma – mass spectrometry (ICP – MS) is a technique that combines an ICP (e.g. argon plasma torch) with a mass spectrometer. It was used not only because it was a comprehensive technique for all our target elements, but it was one of the most sensitive techniques available at the moment. The sample is pumped into the system, it is nebulized and the produced droplets go through the plasma at high temperature (~6000 °C), the solvent evaporates, the resultant solids vaporize, and their elements are thus ionised. Then the ions are separated and finally, they are detected in a mass spectrometer (Perez-Arantegui and Laborda, 2018).
- Isotope ratio mass spectrometry (IRMS). This technique is used to determine isotopic abundances, requiring thus high sensitivity, and needs specialized instrumentation: a multi-collector magnetic sector mass spectrometer, i.e. IRMS (Muccio and Jackson, 2009). Reason why it was used. The IRMS can be used in combination with other instruments. In this case, the isotopic composition of nitrogen from moss tissue and peat samples was the target, so it was used the elemental analyser (EA) coupled with the IRMS. EA-IRMS provides information about the isotopic composition of the whole sample. First, in the EA, the sample is burned in a furnace, then the products are specifically treated, if needed, depending on the isotopes of interest, and finally, they are passed into the IRMS (Muccio and Jackson, 2009). The system works under high vacuum conditions. The ionized samples are then transported to the magnetic

sector mass separator by an ion optical system where they are separated according to the mass/charge ratio (Perez-Arantegui and Laborda, 2018).

1.4.3 Flow injection analysis

Flow injection analysis (FIA) is an automated method in which a specific amount of liquid sample is introduced into a system that has a continuous flow stream (carrier; Ruzicka and Hansen, 1988). The carrier transports the sample (analyte) through a detector that determines the amount of analyte. In this thesis, the flow injector analyser used was equipped with a flow-through absorbance detector. The FIA was based on a colorimetric technique. Ammonia, the target component of the sample studied using this method, was determined by heating with different chemicals in an alkaline buffer. EDTA was used to prevent precipitation of magnesium and calcium. And to improve sensitivity sodium nitroprusside was added. The absorbance of the product of this mixture is measured at 660 nm giving a signal that is proportional to the concentration of ammonia in the sample (American Public Health Association et al., 2017). This method was used because of its sensitivity (0.002 mg N/L) and extremely high response (90 seconds per sample), so it was the most adecuate for the type of samples for analysis (peat and pore water).

1.5 Research aims and objectives

Peatlands and temperate forests are C and N sinks, and thus they reduce the amount of greenhouse gasses (GHGs; e.g. CO_2, N_2O) present in the atmosphere. These habitats are N limited playing BNF a critical role for N nutrition. However, since the industrial revolution, Nr has also been produced anthropogenically through burning fossil fuel or the fertilization

of croplands, before it is re-deposited into the land surface. BNF is an energy costly process, so high rates of Nr deposition may reduce or even shut down BNF activity. The present UK Nr deposition rates range from <10 to >26 kg N ha^{-1} yr^{-1}, even though they have been reduced significantly during the past decades, and it is not clear whether these chronic Nr deposition rates suppress BNF or not, so field studies are needed for a better understanding. Moreover, important controls of BNF activity such as temperature, moisture, or GHGs are changing due to climate change and anthropogenic pollution. Studying the abiotic factors that affect BNF will allow anticipating the response of BNF to future changes in climate and atmospheric pollution. Due to the above, the main aim of the research was to investigate the impacts of atmospheric reactive nitrogen deposition upon rates of biological nitrogen fixation in peatlands and temperate forests.

The specific objectives of this research were:

1. To establish a more robust and direct measurement of BNF in natural ecosystems (Chapter 2).

   Within this objective, the following hypothesis was tested:

   - Acetylene reduction assay underestimates BNF rates.

2. To evaluate the effects of increased atmospheric Nr deposition on rates of BNF in peatlands, and examine the main abiotic factors controlling BNF in the field (Chapter 3).

   The following hypotheses were tested under this objective:

   - Chronic increased atmospheric Nr deposition shuts down BNF.
   - An increase in temperature, pH, light, and moisture increases BNF rates.
   - A decrease in electrical conductivity, dissolved oxygen, and Nr (contained in the form of NO_2^-, NO_3^-, or NH_4^+) in pore water increases BNF rates.

3. To assess the effects of selected macro and micronutrients on BNF in the field (Chapter 3) and through laboratory experiments (chapter 4).

   Under this objective, the following hypotheses were tested:

   - Decades of experimental fertilization in the form of high rates of Nr shuts down BNF.
   - Under chronic rates of increased Nr deposition, a higher availability in selected macro and micronutrients and microbial respiratory metabolites (CO_2, CH_4 and N_2O) increases BNF rates.

4. To investigate the effects of elevated CO_2 on BNF in a temperate mature deciduous forest (Chapter 5).

   The following hypothesis was tested within this objective:

   - In a temperate mature forest, fumigation with CO_2 increases BNF rates for trees to meet plant N demands

1.6 Thesis structure

This thesis comprises 6 chapters. The core chapters (Chapter 2-5) follow a paper-style format because they stand as independent pieces of research. In each chapter, the relevant literature is reviewed, and the methodology is explained, although to avoid repetitions, when the same method was used, it was described briefly, and the reader referred to the relevant chapter for more details. In fact, there is a common research method that links all the chapters which is the $^{15}N_2$ assimilation method. Chapter 1 provides a general introduction and a general literature review about the subject matter of this thesis, as well as the main aim of the research project and specific objectives, and the thesis structure. Chapter 2 is a study on the two main methods to measure BNF that provides a comprehensive overview of the

methodological approach. The results of this chapter were accepted for publication in a research article (Saiz et al., 2019). Chapter 3 investigates BNF measured in the field across an Nr deposition gradient, including the main abiotic factors affecting BNF. Additionally, a portion of the chapter is a study about the effects of decades of N, S, and temperature treatments on BNF. Chapter 4 examines the effects of macronutrient (P and K) additions on BNF activity under N saturation, as well as the effects of the addition of micronutrients (Mg) and CH_4, N_2O, and CO_2 on BNF under N, P, and K saturation. Chapter 5 investigates the effects of elevated CO_2 on BNF activity in feather mosses and soil of a mature temperate forest. Chapter 6 provides a summary of the key findings of the research and indicates further research directions.

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CHAPTER 2: BIOLOGICAL NITROGEN FIXATION IN PEATLANDS: COMPARISON BETWEEN ACETYLENE REDUCTION ASSAY AND $^{15}N_2$ ASSIMILATION METHODS*

2.1 Abstract

Biological Nitrogen Fixation (BNF) is an essential microbial process supplying available nitrogen (N) to *Sphagnum* mosses in ombrotrophic peatlands. Acetylene Reduction Assay (ARA) and the $^{15}N_2$ assimilation are the main methods used for the measurement of BNF. ARA is used as a proxy where the moles of ethylene (C_2H_4) produced from acetylene (C_2H_2) during incubation of mosses and peat are used to estimate the moles of N being fixed using a conversion factor (CF), thus relating the moles of C_2H_4 produced to the moles of N fixed. A theoretical CF of 3:1 originally developed for agricultural soils using pure nitrogenase enzymes is in use; in some cases, a site-specific CF is determined through parallel incubation of mosses and peat with ARA and $^{15}N_2$ assimilation methods to enable the application of ARA for subsequent BNF measurement at high resolution and low cost. However, in recent literature, the reported site and/or species-specific CF for peatlands varies by an order of magnitude, thus raising the question if measured CFs are robust and consistent enough for the estimation of BNF in peatlands. Thus, BNF was measured using the ARA and the direct

$^{15}N_2$ assimilation methods in three different peatlands across the UK during the growing season over two years. The incubations were carried out in parallel on the dominant *Sphagnum* spp. (*S. cuspidatum, S. fallax, S. capillifolium*, and *S. papillosum*) and top bulk peat (0–15 cm). Additional incubations were performed using the direct $^{15}N_2$ assimilation method with and without C_2H_2 addition to evaluate if C_2H_2 was suppressing N assimilation through BNF all together in peatlands. According to the results, the CF varied from 0.001 to 5.363, with a median CF of 0.028 for both mosses and peat, which is far lower than the theoretical 3:1 CF. The CF was also highly variable with differences up to 3 orders of magnitude across the different *Sphagnum* species. The measured CF between years for the same species and across the three peatland sites varied significantly suggesting an inconsistent performance of ARA against the ^{15}N assimilation method. The generally low but highly varied CFs measured under this study shows that C_2H_2 differentially interferes with the activity of diazotrophic microbes, which results in an inconsistent CF at species, and site scales, and over time. In conclusion, ARA is not suitable as a proxy method for estimating and/or modelling BNF in peatlands.

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2.2 Introduction

Biological nitrogen fixation (BNF) is an essential microbial process for the provision of available nitrogen (N) to plants in nutrient-poor ombrotrophic peatlands that otherwise rely on atmospheric deposition of reactive N (Berg et al., 2013; Bragina et al., 2013). Ombrotrophic peatlands are usually dominated by *Sphagnum* spp. (mosses), which are adapted to acidic and nutrient-poor conditions (van den Elzen, et al., 2018). Moss-associated cyanobacteria and free-living diazotrophic bacteria fix atmospheric N_2 into the bioavailable NH_4^+ form, thus enabling the plants to meet their N demands for capturing atmospheric carbon (Postgate, 1982). Nitrogenase enzyme in the diazotrophs is responsible for reducing N_2 into ammonium as below (Smith and Gallon, 1993):

$$N_2 + 10H^+ + 8e^- \rightarrow 2NH_4^+ + H_2\,(16\ ATP) \qquad \text{(Equation 2-1)}$$

There are two main methods for measuring BNF: direct $^{15}N_2$ assimilation ($^{15}N_2$ method) and acetylene reduction assay (ARA; Sprent, 1979; Bellenger et al., 2014). The $^{15}N_2$ method allows for the direct quantification of BNF rates; however, the high cost of isotopic tracing is prohibitive for a widespread repeated use. It involves the incubation of samples with $^{15}N_2$ gas, for direct measurement of ^{15}N incorporation into biomass by N fixers, followed by the determination of ^{15}N signature in the incubated samples through mass spectrometry (Zehr and Montoya, 2007). Although it is a robust method, some practical problems have been reported when using this method. For example, incubation chamber leakage (Chalk et al., 2017), oxygen depletion during long term incubation (over weeks) (Myrold et al., 1999), incomplete and slow equilibration between the $^{15}N_2$ gas and the water sample (Großkopf et al., 2012), and $^{15}N_2$ gas contamination (Dabundo et al., 2014) can result in under or overestimation of BNF.

The ARA is the most common method for measuring BNF in different ecosystem types. It is based on the nitrogenase enzyme preferential reduction of acetylene (C_2H_2) to ethylene (C_2H_4) that involves just two electron transfer, instead of reducing N_2 which involves eight, when C_2H_2 is present at relatively high concentrations (10% v/v; Koch and Evans, 1966; Schöllhorn and Burris, 1967; Hardy et al., 1968), according to the following equation (Bergersen, 1970; Staal et al., 2001):

$$C_2H_2 + 2H^+ + 2e^- \rightarrow C_2H_4 \qquad \text{(Equation 2-2)}$$

Despite its simplicity ARA is an indirect method and a conversion factor (CF) is needed to estimate BNF rate equivalents based on the number of moles of C_2H_4 produced. The theoretical CF obtained from the equations (2-1) and (2-2) relating the number of reducing equivalents is 4:1 (moles of C_2H_4 produced per mole of nitrogen fixed) (Zehr and Montoya, 2007). Empirical measurements in *in vitro* experiments of nitrogen fixing bacteria (*Azotobacter* and *Clostridium*) as well as *in situ* have found that the ratio of C_2H_4 produced to N fixed was between 3 and 4.5 (Hardy et al., 1968). However, some authors consider that a couple of electrons and protons are used to release one molecule of H_2 in equation 1, and therefore, the theoretical CF should be 3:1, which is what has been traditionally used in the soil literature (Postgate, 1982). Many authors have reported deviations from the theoretical CF when measuring BNF in peatlands (Chapman and Hemond, 1982; Schwintzer, 1983), in the laboratory with forest soil cores (Nohrstedt, 1983), or in different nitrogen-fixing systems (Bergersen, 1970). As a result, these authors strongly recommended site-specific calibration of the ARA method using the $^{15}N_2$ method (Bergersen, 1970; Roskoski, 1981; Nohrstedt, 1983) for subsequent application of ARA at large scale over time.

Although there have been several studies relating ARA and the $^{15}N_2$ method in legumes and laboratory cultures (Bergersen, 1970), in forests (Roskoski, 1981; Nohrstedt, 1983), and in

arctic habitats (Liengen, 1999), the effect of C_2H_2 on diazotrophic microbial activity in peatlands and hence BNF has been overlooked. It is known that C_2H_2 interferes with different microbial processes typical of peatlands such as nitrification, blocking it; denitrification, inhibiting the respiratory reduction of N_2O to N_2 (Ryden, 1982); and methanotrophy, inhibiting the oxidation of methane (Kip et al., 2010). These metabolic processes provide energy to diazotrophs (Raghoebarsing et al., 2005) and substrate (for example, coupled and/or direct respiratory N_2O reduction and N fixation; Desloover et al., 2014; Farias et al., 2013), thus their inhibition or suppression in the presence of C_2H_2 might affect C_2H_4 production and hence estimation of BNF rates given that the presence of these microbes in the incubated media (mosses and peat) may vary over time (Raghoebarsing et al., 2005). In recent literature, it has been shown that methanotrophs play an important role in BNF (Larmola et al., 2014; Vile et al., 2014), and that they are present in association of *Sphagnum*-mosses other than cyanobacteria as a key N fixing microbe (Larmola et al., 2010) in peatlands all over the world (Kip et al., 2010).

Many studies have applied site specific CFs in peatlands where *Sphagnum* spp. were present such as 3.11 (Kox et al., 2016), 0.85 (Stewart et al., 2011) or 0.32 (Vile et al., 2014) for mosses, and 1.1 (Knorr et al., 2015) for peat, albeit all of them reported high variability, which raises the question if the site-specific CF is reliable and reproducible over time. In other cases, ARA is applied for *Sphagnum* mosses using the theoretical 3:1 CF (Rousk et al., 2018) or using a CF previously reported for the site (Rousk et al., 2015), whilst indicating that BNF rates for the sites would be underestimated because of the inhibitory effects of C_2H_2 on methanotrophs. The only study that focused on the effects of ARA on BNF rates estimation (Warren et al., 2017) was on peat soil, and therefore, no information exists on the effect of C_2H_2 on diazotrophic microbes associated with *Sphagnum* mosses including cyanobacteria and hence the usefulness of ARA as a proxy of BNF in peatlands.

The main aim was to evaluate the usefulness of the BNF method in peatlands. In this study, ARA was calibrated against the $^{15}N_2$ assimilation method with the objectives to assess: (1) if the conversion factor is consistent across *Sphagnum* species and peat at each site, across sites, and across wider geographic temperate peatland regions, (2) if the CF is consistent over time, and (3) if $^{15}N_2$ assimilation through BNF is completely suppressed in the presence of C_2H_2. Knowing the reported interference of C_2H_2 with microbial activities that have a direct and/or indirect bearing on BNF activity such as methanotrophy, respiratory reduction of N_2O and nitrification (Larmola et al., 2014; Desloover et al., 2014; Farias et al., 2013; Ho and Bodelier, 2015; Sgouridis et al., 2016), it was hypothesized that ARA as a proxy method will underestimate BNF rates in peatlands.

2.3 Material and methods

2.3.1 Study sites and sampling

Sphagnum mosses and peat samples were collected from three ombrotrophic peatlands in the UK: Migneint (52º 59' 20.8" N – 3º 48' 09.8" W) in Wales, Fenn's and Whixall (52º 55' 20.8" N – 2º 45' 58.6" W) in England, and Forsinard (58º 23' 42.2" N – 3º 56' 47.0" W) in Scotland (Fig. 2.1). These sites had different characteristics regarding mean annual temperature, rainfall, and atmospheric reactive nitrogen (Nr) deposition rates (Table 2.1) so that the comparative performance of the two methods could then be representative at large geographic scale.

Moreover, the selected sites were exposed to variable atmospheric Nr deposition and the rationale for the selection was also to evaluate the comparative performance of the two techniques and to know if chronic Nr deposition might be affecting the CF given that a recent paper reported suppression of BNF in feather mosses exposed to high Nr deposition

(Ackermann et al., 2012, Rousk and Michelsen, 2016). Two sampling campaigns were undertaken at Migneint and Fenn's and Whixall sites during the growing season, in 2016 and 2017, respectively; and one campaign at Forsinard in 2017 for *in situ* method performance incubations. Additionally, at Migneint site, samples were collected in spring 2016 for laboratory-based CF determination.

Figure 2.1. Location of the study sites in the United Kingdom.

The vegetation in these sites consisted of mosses and ericoid shrubs. For moss associated BNF quantification, samples of four dominant *Sphagnum* species were collected at each site: *Sphagnum cuspidatum* and *Sphagnum fallax* (most common in hollows); and *Sphagnum papillosum* and *Sphagnum capillifolium* (in hummocks). During the 2016 campaigns, bulk peat (0-15 cm) was also collected from hollows and hummocks, while in 2017 peat was collected only from hollows. *Sphagnum* and peat samples were collected from five random locations within each site to capture the wider inherent spatial variability of each site.

Table 2.1. Mean annual temperature, precipitation and reactive nitrogen (Nr) deposition in the study sites.

Site	**Mean annual temperature (°C)**	**Mean annual precipitation (mm)**	**Atmospheric Nr deposition (kg N ha^{-1} yr^{-1})**
Forsinard (Scotland)	6.9	1104	6
Migneint (Wales)	7.3	2236	17
Fenn's and Whixall (England)	9.5	747	26
Source: Met Office, Air Pollution Information System (APIS).			

2.3.2 $^{15}N_2$ assimilation method

For each species and peat four out of five replicates were incubated with $^{15}N_2$ (98 atom% Cambridge Isotope Laboratories Inc., USA), with the fifth being the control (incubated using ambient air). Each replicate consisted of 20 live moss shoots (~ upper 5 cm) of the selected moss species or 10 g of peat after passing it through a 2 mm sieve. Shoots and peat were placed in 50 ml serum vials which were capped with air tight rubber septa. Following the closure, 5 ml headspace air was drawn using gas tight syringe and replaced with 5 ml of the $^{15}N_2$ (98 atom%) gas (10% headspace concentration) and the bottles were then placed 'upside down' (avoiding cap shade) in the same area from where the samples were collected and incubated for 24 hours to avoid issues of oxygen depletion during long-term incubation (Myrold et al., 1999). In case of peat, samples were placed under the moss carpet (dark conditions). Parallel incubations were run for ARA (details below). Additionally, in order to

control some of the main factors affecting BNF (temperature, light), one set of moss and peat samples from Migneint were incubated under laboratory conditions with the temperature set at 20 ºC (± 2) and the light/dark cycle of 12 hour, while maintaining light intensity through artificial light (photosynthetically active radiation of ~2000 μmol m^{-2} s^{-1}) to determine CF under optimal laboratory conditions for comparison with field-based incubations.

After the 24-hour incubation, the vials were opened and aerated to flush out any remaining $^{15}N_2$ gas and bring it to ambient conditions. Immediately after aeration, the samples were transported in a cool box to the laboratory and then weighed and dried at 70 ºC for 72 hours. Dried samples were manually pulverised (<2 mm) and subsamples (~ 1 g) were sent to the Life Sciences Mass Spectrometry Facility at the Centre for Ecology and Hydrology, Lancaster for ^{15}N content analysis by Isotope Ratio Mass Spectrometry (IRMS) using a Carlo Erba NA1500 (Italy) elemental analyser coupled to a Dennis Leigh Technologies (UK) isotope ratio mass spectrometer. In-house working standards of natural abundance (flour and soil) were analysed every twelfth sample giving an analytical precision of 0.36 ‰. They were calibrated against the certified reference material IAEA-N1 (NIST number 8547, National Institute of Standards and Technology, Gaithersburg, USA). All the control and enriched moss and peat samples were analysed in duplicate (Jardine and Cunjak, 2005) and their variability was within the limits of the analytical precision of the IRMS. They were also analysed duplicates of three non-enriched tissues samples on the IRMS and the resulting reproducibility of the analysis including a cross-laboratory check (details in section 2.3 below) was within the analytical precision of the IRMS. Subsequently, only one control and four enriched incubations were run during BNF measurements while ensuring that the duplicate runs of each sample on the IRMS was within the analytical precision limits. This check was critical given that the experiment relied on the incubation of one non-enriched

sample in the field for calculating BNF rates using the following formula (Equation 2-3; Liengen, 1999):

$$Y = \left(\frac{atom\%\ ^{15}Nexcess}{100}\right) x \left(\frac{totalNsample\ x\ 10^{9}}{t\ x\ 28}\right) x \left(\frac{100}{\%^{15}N\ headspace}\right)$$

where Y (nmol N g^{-1} dw h^{-1}) is the amount of N fixed during the experiment, atom% $^{15}Nexcess$ is the difference between $atom\%^{15}Nsample$ and $atom\%^{15}Ncontrol$, total N is the total amount of nitrogen in the sample (g N 100 g^{-1} dw), t is the incubation time, 28 is the molecular weight of N_2 (g mol^{-1}), and $\%^{15}N$ is the percentage of ^{15}N out of the total amount of N gas in each incubation bottle.

2.3.3 $^{15}N_2$ Gas quality control

Contamination of commercial $^{15}N_2$ gas with ^{15}N-labelled nitrate and ammonium can interfere with the detection of BNF (Dabundo et al., 2014). The potential contamination of the $^{15}N_2$ gas as well as the possibility of abiotic uptake of $^{15}N_2$ gas were evaluated by incubating six samples of dried (105ºC) mosses for 24 hours, three with $^{15}N_2$ enriched gas as above and three without. After the incubation, the samples were processed as above and were sent to two different laboratories (CEH Lancaster and Bristol University) for ^{15}N analysis using IRMS to ensure cross laboratory checks (Bahlmann et al., 2010). The results obtained (average $\delta\ ^{15}N$ in sample of enriched ones: -0.360; and of non-enriched ones: -0.387) showed a difference of -0.03 $\delta\ ^{15}N$ between the treatments. Therefore, this averaged difference (-0.03 $\delta\ ^{15}N$) was used as a threshold below which any difference between the control and enriched samples incubated for direct BNF measurement was not considered.

2.3.4 Acetylene reduction assay (ARA)

Following the placement of 20 shoots of mosses or 10 g of field moist peat in serum bottles (n = 5) and capping with septa, 10 % of the headspace was replaced with (10 % v/v) pure and fresh C_2H_2 obtained by adding deionised water to calcium carbide. Immediately after doing so, a 3 ml gas sample was obtained (T_0), and replaced by the same gas mixture to maintain atmospheric pressure within the vials. Gas samples were subsequently collected following the same procedure at 6 and 24 hours.

To check for possible contamination of the acetylene gas with ethylene and endogenous production of ethylene by mosses and peat, quality control incubations were carried out. Mosses and peat were incubated with and without the addition of C_2H_2 (each with three replicates), whereas three bottles received C_2H_2 but no sample and three bottles were incubated under ambient air without sample and C_2H_2. The results showed no endogenous C_2H_4 production or gas contamination, and negligible level of C_2H_4 in air was detected which was later used for background correction while calculating ethylene production rates.

The gas samples were analysed for C_2H_4 concentration using a gas chromatograph (Varian 39000) equipped with a Restek-Alumina BOND/MAPD column (30 mm x 0.53 mm x10 µm) and a flame ionization detector (FID) using He as a carrier gas. The temperatures of the injector and detector were 200 ºC, and for the column was 135 ºC. The head pressure was 3.4 psi and the carrier flow 3.2 ml min^{-1}. The injection was manual. C_2H_4 production rates were calculated by linear regression between time intervals T_0-T_{24} using a standard calibration curve for each of the daily batch samples. Using standards injection after 10 samples each, the quality of the runs was checked and where needed, corrected for any drift in the signal.

2.3.5 ARA – $^{15}N_2$ direct assimilation conversion factor (CF ratio)

The ARA conversion factor was calculated by dividing moles of C_2H_4 produced (ARA method) by the moles of N fixed ($^{15}N_2$ direct assimilation) for each parallel incubation for different species and peat collected from the Migneint site and incubated under laboratory conditions (Vile at al. 2014) in 2016. Following the CF determination under laboratory conditions, CFs were then estimated for the field-based incubations for the Fenn's and Whixall (2016-17), Migneint (2016-17), Forsinard (2017) sites. CFs were calculated per site as well as per species and peat type within each site.

2.3.6 BNF determination with $^{15}N_2$ assimilation method with and without C_2H_2 addition

In 2017, during the growing season, samples (*Sphagnum* mosses and peat) collected from Fenn's and Whixall, Migneint, and Forsinard sites, were incubated for BNF measurement using the $^{15}N_2$ assimilation method as described above, where 3 replicates were further amended with C_2H_2 (10 % v/v) and 3 without to evaluate if the presence of C_2H_2 will completely inhibit N_2 reduction to NH_4^+ by diazotrophs given that under high C_2H_2 concentration, diazotrophs have been shown to preferentially reduce C_2H_2 than reducing N_2 (Koch and Evans, 1966; Schöllhorn and Burris, 1967).

2.3.7 Statistical analysis

Data were tested for normality and homogeneity of variance and since they were found non-normal and non-homogeneous, only non-parametric statistical tests were applied. To test differences in paired samples, bootstrapped t-test was used. Differences in C_2H_4 production

(ARA) and BNF ($^{15}N_2$ method) and the effect of C_2H_2 on BNF rates were tested using the Wilcoxon ranked sum test. To evaluate the effect of the site and the species it was used the Kruskal-Wallis test. All the analysis was performed on the IBM SPSS Statistics program, version 24.

2.4 Results

2.4.1 Conversion factors

The CFs obtained showed great variability across species, sites and time ranging from 0.001 to 5.363 moles of C_2H_4 produced per mol of N fixed (Table 2.2). The mean (± standard deviation) obtained for all the species and sites is 0.45 ±2.373, while the median (± median absolute deviation) is 0.028 ±0.022 (see supplementary Table 2.S1) which is far lower than the theoretical ratio 3:1. Across *Sphagnum* species within sites and between years, the CF varied orders of magnitude (Table 2.2). CF values in peat also differed substantially between laboratory-based, in situ incubations in 2016 and 2017 at the Migneint site, while in case of Fenn's and Whixall and Forsinard the data was <LOD except in once instance (Table 2.2). Overall, the available data suggest high variability in CF values of peat between sites and years (Table 2.S1). In case of *S. cuspidatum* extreme CF values were observed during the laboratory incubations in 2016 from the Migneint site, *in situ* incubation at the Migneint site in 2016, and *in situ* incubations at the Forsinard site in 2017, which resulted in variations up to three orders of magnitude. Exclusion of these extreme CF values still resulted in significant differences where the median CFs by species and year and between lab-based and *in situ* incubations exhibited variations of up to two orders of magnitude (see supplementary Table 2.S2).

Table 2.2. Conversion factors obtained as the ratio between C_2H_4 production measured by the ARA method divided by the N fixed using the $^{15}N_2$ method. Data shown for species (rows) is the median (± median absolute deviation - MAD) of the three replicates (four for Migneint and Fenn's and Whixall 2016). Mean conversion factors (± standard deviation) for the *Sphagnum* species and peat are also shown. LOD: under the limit of detection. ND: no data.

	Site	**Lab incubations Migneint**	**Migneint**		**Fenn's & Whixall**		**Forsinard**	**Median of the three sites for the different species (±MAD)**
	Name / Year	**2016**	**2016**	**2017**	**2016**	**2017**	**2017**	**2017**
Mosses	*S. cuspidatum*	0.457 (±0.071)	5.363 (±1.740)	0.056 (±0.014)	0.002 (±0.001)	0.002 (±0.001)	0.114 (±0.003)	0.056 (±0.054)
	S. capillifolium	0.18 (±0.087)	0.025 (±0.011)	0.010 (±0.0002)	0.035 (±0.005)	0.002 (±0.001)	0.010 (±)	0.010 (±0.0002)
	S. fallax	0.053 (±0.035)	0.047 (±0.020)	0.036 (±0.004)	0.033 (±)	0.005 (±0.002)	0.008 (±0.008)	0.008 (±0.003)
	S. papillosum	0.015 (±0.015)	0.014 (±0.008)	0.043 (±0.003)	0.010 (±0.003)	0.031 (±0.020)	0.094 (±0.026)	0.043 (±0.012)
	Median (±MAD)	0.095 (±0.084)	0.045 (±0.036)	0.039 (±0.010)	0.012 (±0.011)	0.005 (±0.003)	0.081 (±0.060)	0.026 (±0.018)
	Mean (±SD)	0.205 (±0.252)	1.989 (±5.334)	0.091 (±0.191)	0.018 (±0.016)	0.010 (±0.015)	0.203 (±0.323)	0.089 (±0.089)
Peat	Peat hollows	0.001 (±0.0001)	0.011 (±0.007)	LOD	LOD	LOD	LOD	
	Peat hummocks	0.005 (±0.004)	0.027 (±0.022)	ND	0.020 (±0.013)	ND	ND	
	Median (±MAD)	0.001 (±0.0002)	0.018 (±0.010)		0.020 (±0.013)			
	Mean (±SD)	0.004 (±0.005)	0.019 (±0.019)		0.020 (±0.018)			

BNF rates measured by the $^{15}N_2$ method and the ARA method showed significant differences. The median ARA rates of mosses and peat per sites in 2017 estimated by applying the theoretical CF of 3:1 for the C_2H_4 produced during parallel incubations, were significantly lower (Fig. 2.2; C_2H_4 produced also shown) than the direct BNF rates measured based on the $^{15}N_2$ assimilation method. The difference between the median BNF rates based on ARA and ^{15}N assimilation measurements was more prominent for the Fenn's and Whixall site (ARA being four hundred times lower than $^{15}N_2$ method). Moreover, by applying the median field-based CF obtained for each of the sites, the BNF rates obtained by the ARA method were also relatively lower than the ones obtained using the $^{15}N_2$ assimilation method (Fig. 2.2), which suggest an important underestimation of BNF by even applying the species, peat and site-specific CFs for the sites.

BNF measured by the $^{15}N_2$ assimilation method was higher than the ARA (C_2H_4 produced, no CF applied) for each *Sphagnum* species in each site, albeit the difference varied widely among species (Fig. 2.3). At Migneint the ^{15}N assimilation rate was 20 (*S. cuspidatum*) to 98 (*S. capillifolium*) times larger than the C_2H_4 production; at Fenn's and Whixall it was 54 (*S. papillosum*) to 271 (*S. cuspidatum*) times larger; and at Forsinard it was 7 (*S. capillifolium*) to 50 (*S. fallax*) times. Note that no detectable fixation was found in peat. This large range in the ratio of ^{15}N fixed to C_2H_4 produced signify a lack of consistency by species and sites.

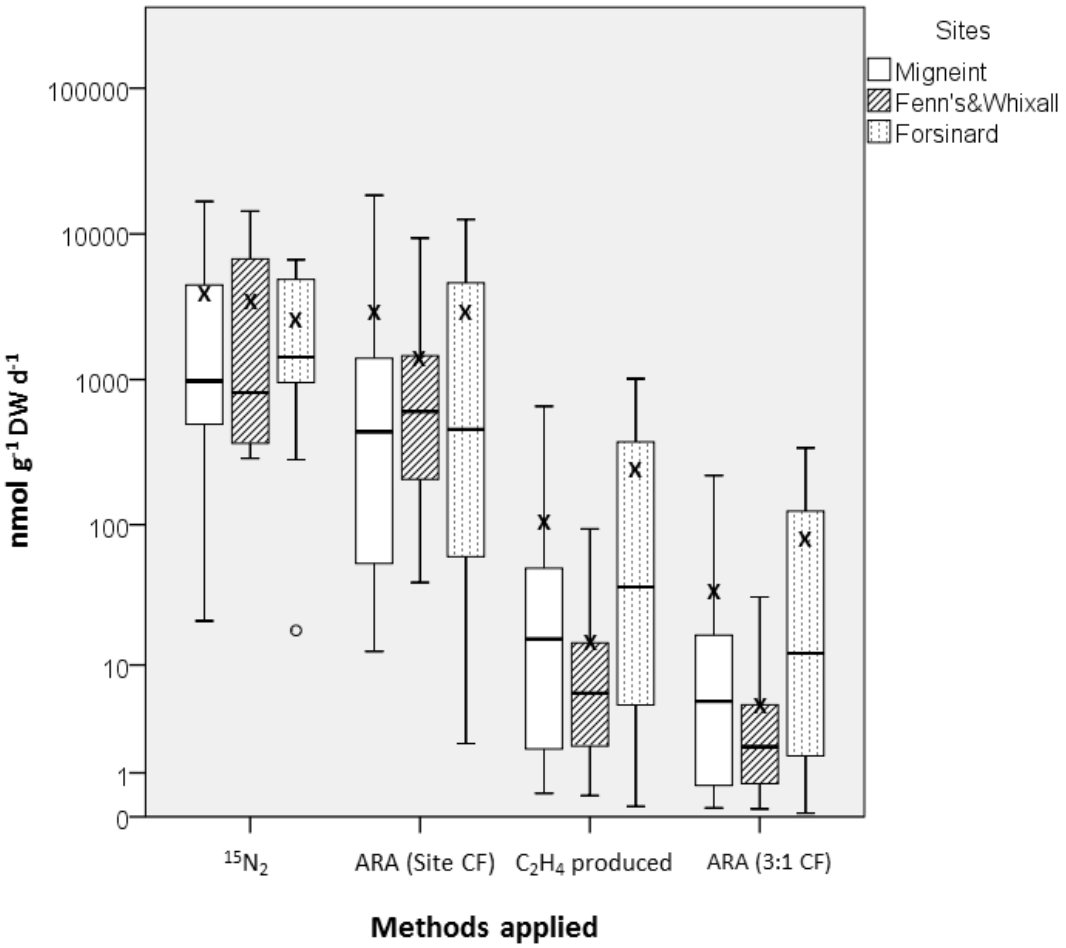

Figure 2.2. Rates of C_2H_4 produced (C_2H_4 nmol g^{-1} DW d^{-1}), BNF rates estimated (nmol N g^{-1} DW d^{-1}) using the theoretical 3:1 CF of ARA method, BNF rates estimated (nmol N g^{-1} DW d^{-1}) using site specific CFs (0.039 for Migneint; 0.005 for Fenn's & Whixall; and 0.081 for Forsinard), and direct BNF rates measured using $^{15}N_2$ assimilation method (nmol N g^{-1} DW d^{-1}) at each site in 2017. Solid lines within the boxes indicate medians, the x indicates the mean value, the top of the box marks the 75th percentile, and the bottom the 25th percentile. The whiskers mark the maximum, upper part, and the minimum, lower part, values, and the dot is an outlier (<1.5 IQR). Note the log-scale y-axis (n=15).

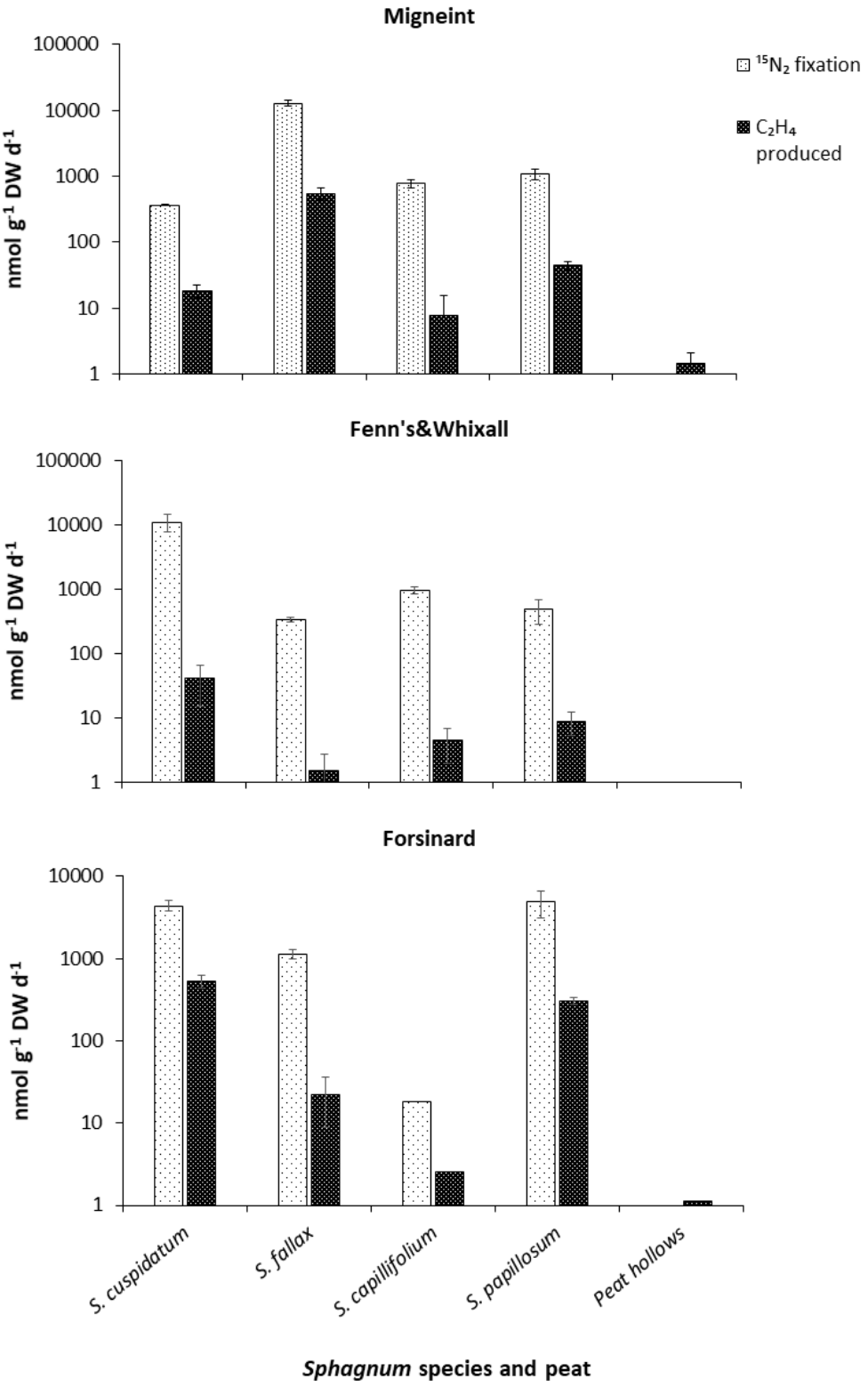

Figure 2.3. Estimated C_2H_4 produced (C_2H_4 nmol g^{-1} DW d^{-1}) using ARA method and direct BNF measurements (nmol N g^{-1} DW d^{-1}) using $^{15}N_2$ assimilation method, for each of the *Sphagnum* species and peat within each site in 2017. Shown are the median values, ± median absolute deviation (n=3). No bars mean no N_2 fixation or no C_2H_2 reduction. Note the different log-scale y-axis.

2.4.2 BNF by $^{15}N_2$ method with and without C_2H_2

C_2H_2 failed to completely inhibit N_2 reduction to NH_4^+ using the ^{15}N uptake as a direct evidence (Fig. 2.4). The partial suppression of BNF by the C_2H_2 was also inconsistent across the three sites compared to BNF rates in the absence of C_2H_2. The relative percentage of BNF suppression calculated at each site (Suppression % = [(BNF with C_2H_2 − BNF without C_2H_2) / BNF without C_2H_2] * 100) based on the mean was 74% at Migneint, 87% at Fenn's and Whixall, and 99% at Forsinard. However, based on the median, it was found that in Migneint there was no suppression but a relative enhancement of 6%, whilst in Fenn's and Whixall, the relative percentage of BNF suppression was 64% and in Forsinard 99%, showing a marked variability. The differences in the percentage of BNF suppression among species within each site also varied substantially (ranging from none to complete suppression and even enhancements) showing an inconsistent suppression pattern (Fig. 2.S1).

2.5 Discussion

Both for *Sphagnum* spp. and peat, the rates of C_2H_4 produced were lower than the direct assimilation of N determined through $^{15}N_2$ assimilation method. This resulted in extremely low species-specific CFs (Table 2.2) signifying potentially serious underestimation of BNF rates by the ARA method, particularly in *Sphagnum* dominated peatlands. Hardy et al. (1968) reported CF ratios between 3 and 4.5 after empirical measurements of BNF activity in bacteria cultures and nitrogenase enzyme preparations as well as in free-living bacteria using parallel ARA and $^{15}N_2$ direct assimilation methods. Following this publication, the estimated theoretical CF of 3:1 has been applied in various ecosystems including peatlands (Basilier, 1979; Markham, 2009; Rousk et al., 2018). However, many studies have reported

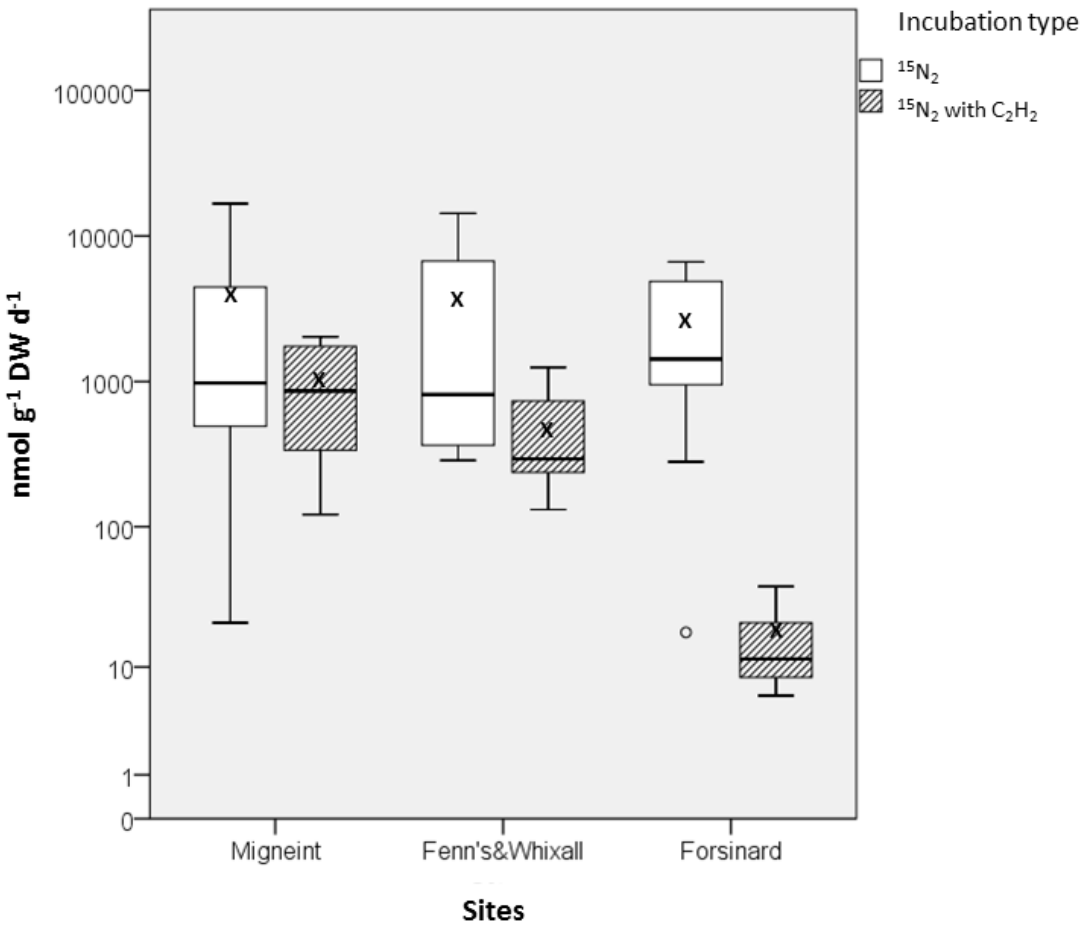

Figure 2.4. Estimated BNF rates (nmol N g^{-1} DW d^{-1}) in *Sphagnum* mosses and peat, by site, in 2017, using the $^{15}N_2$ assimilation method with and without C_2H_2 addition. Note the log-scale y-axis (n=15). See Fig. 2.2 for box plot description.

greater CFs than the theoretical one ranging from 3.11 to 4.5 for peat and *Sphagnum* spp. together (Basilier, 1980: Chapman and Hemond, 1982; Urban and Eisenreich, 1988; Kox et al., 2016; Warren et al., 2017). One potential plausible explanation for the discrepancies over the theoretical CF was the possibility of significant endogenous C_2H_4 production in subsurface peat (Schwintzer, 1983; Kox et al., 2016). Conversely, lower CFs than the theoretical CF have also been reported for *Sphagnum* spp. such as 2.48 (Sorensen et al., 2006), 0.85 (Stewart et al., 2011), 0.32 (Vile et al., 2014), or *Sphagnum* peat 1.1 (Knorr et al., 2015), as well as for bryophytes 0.25 (Menge and Hedin, 2009). Therefore, the reported

site-specific CF of peatlands show marked deviations from the theoretical CF and this is consistent with the range of CF measured under this study, albeit it has been gone further in estimating CFs per species and in different peatlands across an Nr deposition gradient. High rates of Nr deposition did not shut down BNF and it was not detected any effect on the comparative performance of the methods and the resulting calculation of the CFs. The high variability of these measured CFs suggests that the *Sphagnum* microbiome and its species-specific distribution (Kostka et al., 2016), as well as the inhibitory effects of C_2H_2 on microbial processes such as methanotrophy, nitrification and nitrous oxide reduction may be at play, thus leading to highly inconsistent CFs across species, sites and time. It can be speculated that such a differential effect could be responsible for the extreme CF values estimated in case of *S. cuspidatum* (Lab incubations Migneint 2016, Migneint 2016) and hence as a *hot spot* of biogeochemical processes (McClaine et al., 2003).

The variability in the measured CFs in this study is further substantiated by the fact that the presence of C_2H_2 differentially affected the suppression of N_2 fixation, but did not completely suppress it across the sites as demonstrated under pure in vitro incubations of nitrogenase enzymes in the presence of C_2H_2 (Koch and Evans, 1966; Schöllhorn and Burris, 1967). This differential suppression response under the ARA must have led to the variable CF ratios this study estimated. It appears that the diversity of diazotrophic communities from autotrophic cyanobacteria to chemolithotrophic methanotrophs associated with *Sphagnum* mosses in peatlands may have been affected differentially by C_2H_2 with varied C_2H_4 production across species, sites and time. The CF is estimated to save resources for widespread application of ARA in peatlands; however, the difference in CFs at species and site level and over time is not consistent and thus it is recommended the use of ^{15}N assimilation method for measuring BNF in peatlands.

The very low rates of C_2H_4 production by the ARA in this study could be explained by the presence of methanotrophs and the inhibitory effects of C_2H_2 on the methane monooxygenase enzyme in these bacteria; thus depriving the methanotrophs of a significant bacterial energy to fuel BNF (Flett et al., 1975; De Bont and Mulder, 1976). Widespread presence of methanotrophs associated with *Sphagnum* species has been established (Basiliko et al., 2004; Raghoebarsing et al., 2005; Kip et al., 2010; Larmola et al., 2010). Furthermore, Larmola et al. (2014) and Vile et al. (2014) have demonstrated that methanotrophs can contribute up to 40% to total BNF in peatlands, therefore the use of the ARA method could underestimate the BNF rates up to a similar percentage and even more, as shown by this study (~53% on average), which is in agreement with a peat BNF study (~55%) in Minnesota, USA (Warren et al., 2017). C_2H_2 also shuts down nitrification, and stops the reduction of N_2O to N_2 (Ryden, 1982). The inhibition of these processes particularly of nitrification leads to an increase in plant available nitrogen (NH_4^+) that may in turn limit biological nitrogen fixation (Stewart et al., 2013) particularly in ecosystems with a tightly coupled N cycle such as peatlands. Moreover, inhibition of N_2O reduction which could potentially provide energy and substrate for BNF, might also be a factor in affecting C_2H_4 production rates and hence low and inconsistent CF in the end as observed in this study. For example, Farias et al. (2013) and Desloover et al. (2014), reported respiratory reduction of N_2O to N_2 and its subsequent fixation by diazotrophs in pure bacterial cultures and thus inhibition of N_2O reduction to N_2 by C_2H_2 in peatlands might be affecting N fixation rates.

High microbial diversity has been found in *Sphagnum* species between different habitats (e.g. hummocks vs hollows) within peatlands (Opelt et al., 2007). Two main functional groups of bacteria have been studied in *Sphagnum* mosses, nitrogen-fixers and methane-oxidizers (some of which are able to fix nitrogen as well; Auman et al., 2001), and important differences in the community diversity of these two types of bacteria between *Sphagnum*

species have been reported (Bragina et al., 2013). This microbial community diversity could potentially explain the high variability of the CF between *Sphagnum* spp. observed in this study, due to the differential presence of these kinds of bacteria, and the different level of interference in their associated microbiological processes in the presence of C_2H_2. Further microbiological studies are recommended to verify the net inhibitory impact against the abundance and expression of N fixers, methanotrophs, nitrifiers and N_2O reducers.

2.5.1 Conclusions

The conversion factors measured under this study using the direct ^{15}N assimilation and ARA methods were inconsistent across *Sphagnum* species and peat, sites, and over time. This lack of reproducibility and deviation from the theoretical CF of 3:1 show that ARA as a proxy method cannot fully reflect the BNF activity and hence fixation rates in peatlands. This lack of consistency and partial, yet differential suppression of N_2 fixation in the presence of C_2H_2 led to lower CF values and hence underestimation of BNF. Direct interference and/or inhibition of microbes, particularly methanotrophs seem to result in the differential suppression of N_2 fixation. Therefore, caution is needed when estimating and modelling BNF rates based on the ARA method in peatlands. It can be determined that the ARA method is not suitable for BNF measurement in *Sphagnum*-dominated peatlands.

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2.7 Supplementary material

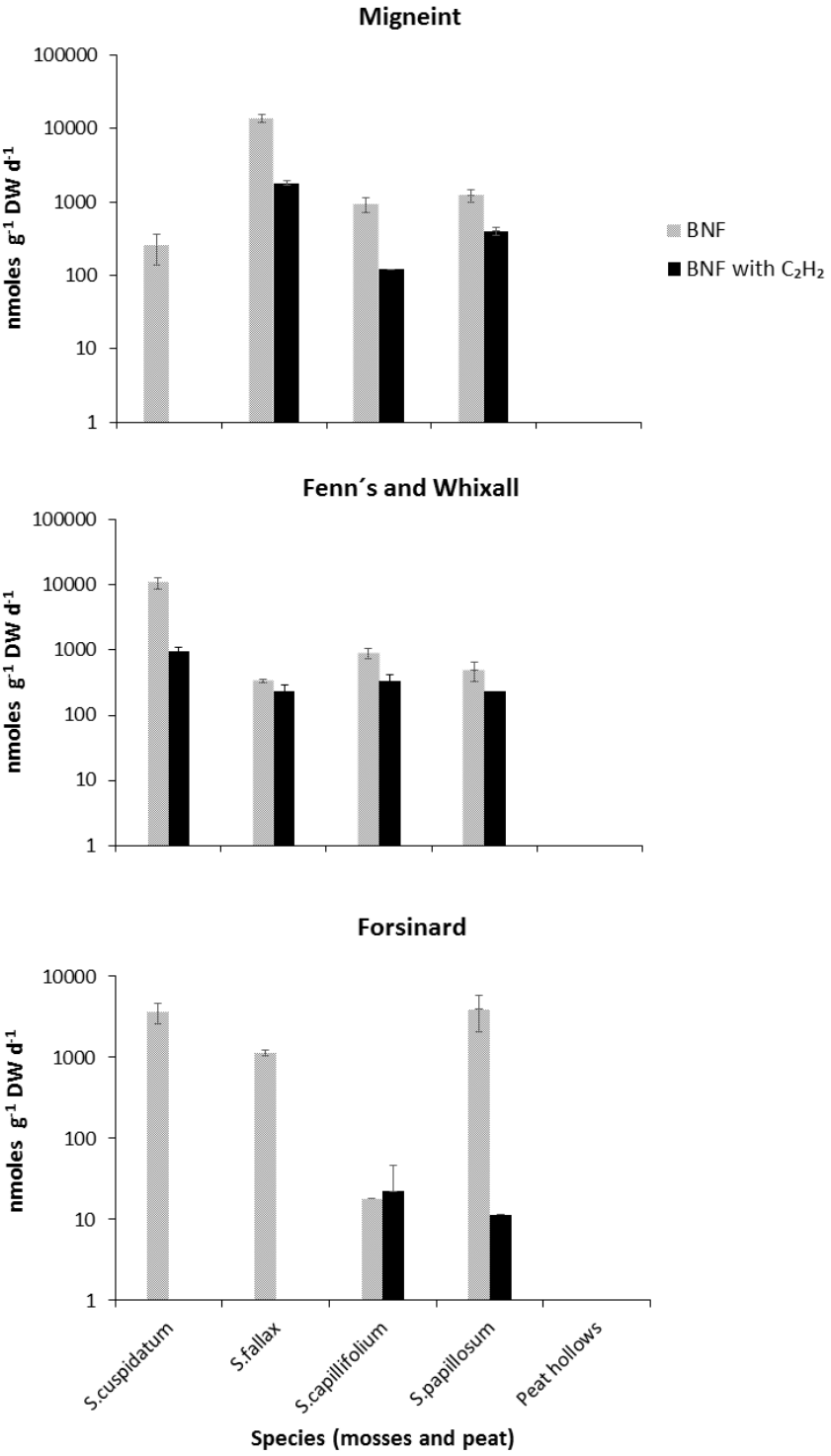

Figure 2.S1. Estimated BNF rates (nmoles N gDW^{-1} d^{-1}) in *Sphagnum* mosses by species and peat at each site with and without C_2H_2 addition. Shown are means ±SE (n=3). Note the different log-scale y-axis.

Table 2.S1. Conversion factors obtained as the ratio between C_2H_4 production measured by the ARA method divided by the BNF measured by the ^{15}N method. Data shown for species (rows) is the median (± MAD, median absolute deviation) of the three (four for Migneint and Fenn's & Whixall 2016) replicates. Mean (± standard deviation) for the *Sphagnum* species and peat are shown as well. LOD.: under the limit of detection. ND: no data. MAD = Median absolute deviation; SD = Standard deviation; ND = No data; LOD = Under the limit of detection.

Site	**Lab incubations Migneint**	**Migneint**		**Fenn's & Whixall**		**Forsinard**
Name	**2016**	**2016**	**2017**	**2016**	**2017**	**2017**
S. cuspidatum	0.4565	3.6230	0.0428	LOD	0.0139	0.6376
	0.8463	20.390	0.6632	0.0006	0.0010	0.1111
	0.3856	LOD	0.0563	0.0018	0.0022	0.1139
	ND	5.3630	ND	0.011	ND	ND
S. capillifolium	0.2707	0.0312	LOD	LOD	0.0024	LOD
	0.0894	0.0097	0.0102	0.0402	0.0063	0.0098
	0.1839	0.0178	0.0098	0.0297	0.0016	LOD
	ND	0.1124	ND	LOD	ND	ND
S. fallax	0.1003	0.0091	0.0356	LOD	0.003	0.0062
	0.0180	0.0481	0.0209	LOD	LOD	0.0076
	0.0531	0.0452	0.0393	LOD	0.007	0.0323
	ND	0.0970	ND	0.0326	LOD	ND
S. papillosum	0.0375	0.0070	0.0289	0.0069	ND	0.9501
	0.0004	0.0048	0.0461	0.0380	0.0507	0.0680
	0.0151	0.02	0.0428	0.0079	0.0117	0.0935
	ND	0.0580	ND	0.0130	ND	ND
Median (±MAD)	0.095 (±0.084)	0.045 (±0.036)	0.039 (±0.010)	0.012 (±0.011)	0.005 (±0.003)	0.081 (0.060)
Peat hollows	0.0008	0.0106	LOD	LOD	LOD	LOD
	0.0009	0.0254	LOD	LOD	LOD	LOD

	LOD	LOD	LOD	LOD	LOD	LOD
	LOD	0.0033	LOD	LOD	LOD	LOD
Peat hummocks	0.0114	0.0056	ND	LOD	ND	ND
	0.0010	0.0487	ND	LOD	ND	ND
	0.0054	LOD	ND	0.0075	ND	ND
	LOD	LOD	ND	0.0332	ND	ND
Median (±MAD)	0.001 (±0.0002)	0.018 (±0.010)		0.020 (±0.013)		
All data median (±MAD)	0.028 (±0.022)					
All data mean (±SD)	0.45 (±2.373)					

Table 2.S2. Conversion factors obtained as the ratio between C_2H_4 production measured by the ARA method divided by the N fixed using the $^{15}N_2$ method. Data shown for species (rows) is the median (± MAD, median absolute deviation) of the three (four for Migneint and Fenn's & Whixall 2016) replicates while excluding the extreme values (identified using the formula ±1.5 intern quartile range step). The mean (± standard deviation) for the *Sphagnum* species and peat is shown as well. LOD.: under the limit of detection. ND: no data. EEV: Excluded extreme values.

	Site	**Lab incubations Migneint**	**Migneint**		**Fenn's & Whixall**		**Forsinard**	**Median of the 3 sites for the different species (±MAD)**
	Name / Year	**2016**	**2016**	**2017**	**2016**	**2017**	**2017**	**2017**
Mosses	*S. cuspidatum*	EEV	EEV	0.050 (±0.007)	0.002 (±0.001)	0.002 (±0.001)	0.113 (±0.001)	0.050 (±0.047)
Mosses	*S. capillifolium*	0.089 (±)	0.025 (±0.011)	0.010 (±0.0002)	0.035 (±0.005)	0.002 (±0.001)	0.010 (±)	0.010 (±0.0002)
Mosses	*S. fallax*	0.053 (±0.035)	0.047 (±0.020)	0.036 (±0.004)	0.033 (±)	0.005 (±0.002)	0.008 (±0.001)	0.008 (±0.003)
Mosses	*S. papillosum*	0.015 (±0.015)	0.014 (±0.008)	0.043 (±0.003)	0.010 (±0.003)	0.031 (±0.020)	0.081 (±0.013)	0.043 (±0.012)
	Median (±MAD)	0.038 (±0.022)	0.026 (±0.019)	0.037 (±0.009)	0.012 (±0.011)	0.005 (±0.003)	0.050 (±0.043)	0.026 (±0.018)
	Mean (±SD)	0.045 (±0.038)	0.038 (±0.036)	0.033 (±0.016)	0.018 (±0.015)	0.010 (±0.015)	0.055 (±0.047)	0.034 (±0.037)
Peat	Peat hollows	0.001 (±0.0001)	0.011 (±0.007)	LOD	LOD	LOD	LOD	LOD
Peat	Peat hummocks	0.005 (±0.004)	0.027 (±0.022)	ND	0.020 (±0.013)	ND	ND	ND
	Median (±MAD)	0.001 (±0.0002)	0.011 (±0.007)		0.020 (±0.013)			
	Mean (±SD)	0.004 (±0.005)	0.019 (±0.019)		0.020 (±0.018)			

CHAPTER 3: EFFECTS OF INCREASED ATMOSPHERIC REACTIVE NITROGEN DEPOSITION UPON RATES OF BIOLOGICAL NITROGEN FIXATION IN PEATLANDS

3.1 Abstract

Biological nitrogen fixation (BNF) by diazotrophic microbes including cyanobacteria represents the natural pathway by which mosses meet the demands for reactive nitrogen (Nr) in pristine rain-fed peatlands. However, there has been an increase in atmospheric Nr deposition due to a growth in fossil fuel burning and the use of agricultural fertilizers. Because BNF is an energy-intensive process, it has been suggested that high Nr deposition loads (> 10 kg N ha^{-1} yr^{-1}) may reduce and even entirely shut down BNF activity in peatlands as Nr availability is no longer limited. To evaluate the response of BNF under chronic Nr deposition extending over decades, BNF rates were measured in three peatlands across an Nr deposition gradient (6, 17, 27 kg N ha^{-1} yr^{-1}) in the UK, and one in Sweden under background Nr deposition (~2 kg N ha^{-1} yr^{-1}). Long term experimental N (15 and 30 kg ha^{-1} yr^{-1}) and sulphur (10 and 20 kg ha^{-1} yr^{-1}) fertilization plots in the Swedish peatland were also evaluated for BNF activity. BNF rates associated with the dominant mosses (*Sphagnum* spp) found in hummocks and hollows as well as bulk peat (0-15 cm) were determined by the

direct $^{15}N_2$ assimilation method. According to the results, BNF activity in the sites receiving Nr deposition rates of 6, 17 and 27 kg N ha^{-1} yr^{-1} was suppressed by 54, 69 and 74%, respectively, compared to the pristine site in Sweden. In general, moss-associated BNF rates were higher in mosses located in hollows than in hummocks. These results show that moss-associated BNF does not completely shut down under chronic Nr deposition. These results were further substantiated by similar observation at the long-term experimental fertilization sites in Sweden, where N and S treatments did not shut down BNF completely. This study demonstrates that the contribution of BNF needs to be accounted for when modelling the N economy of peatlands exposed to chronic Nr deposition.

3.2 Introduction

Nutrient-poor peatlands, often dominated by *Sphagnum* mosses (Lamers et al., 2000; Bragazza et al., 2006), rely on biological nitrogen fixation (BNF) from associated as well as free-living diazotrophic organisms (Vile at al., 2014; Knorr et al., 2015) as an additional source of nitrogen (N) in order to meet their metabolic N demands (Urban and Eisenreich, 1988; Moore et al., 2004). The process of fixing atmospheric N_2 is energy demanding, requiring 16 molecules of ATP to obtain just 2 moles of NH_4^+ (Bellenger et al., 2014):

$$N_2 + 10H^+ + 8e^- \rightarrow 2NH_4^+ + H_2 \text{ (16 ATP)} \qquad \text{(Equation 3-1)}$$

Therefore, high rates of atmospherically deposited Nr could potentially negate the need for a 'costly' investment on BNF. In boreal forest ecosystems, it has been shown that BNF decreased as Nr increased (Leppänen et al., 2013) while it has been suggested that a threshold of 3 kg N ha^{-1} yr^{-1} (Ackermann et al., 2012) or 10 kg ha^{-1} yr^{-1} (Rousk et al., 2014) can inhibit BNF. However, experimental studies with higher than ambient Nr deposition rates (e.g. Gundale et al., 2011) have found lower BNF rates in boreal forests, but not complete inhibition. Similarly, in temperate regions, Nr addition experiments have found lower BNF rates with higher Nr availability in switchgrass fields (Roley et al., 2018) and in peatlands dominated by *Sphagnum* mosses (Kox et al., 2016) but, also unaffected BNF rates on *Sphagnum* mosses in an experiment performed in a temperate peatland (van den Elzen et al., 2018). However, little is known at present on how BNF activity varies under a natural gradient of chronic Nr deposition.

Since the industrial revolution the contribution of anthropogenic Nr to the biosphere has increased enormously because of three principal activities: agricultural intensification,

fertilizer production and fossil fuel combustion (Gundale et al., 2011; Vitousek et al., 2013). In Western countries, the Nr deposition rates are expected to stagnate and decline during the next decades (Payne et al., 2017). In the UK, the Nr deposition rates in peatlands range from <10 kg N ha^{-1} yr^{-1} in the north of Scotland to more than 30 kg N ha^{-1} yr^{-1} in the centre of England (Field et al., 2014). The same study also remarked that it is highly likely that the areas with the lowest rates of Nr such as Forsinard in Scotland will suffer an increased from about 6 kg N ha^{-1} yr^{-1} to 9 kg N ha^{-1} yr^{-1} by 2030.

BNF can also be indirectly affected by Nr deposition via a gradual decay of *Sphagnum* mosses: while at the beginning an increased N supply could result in a greater growth of the *Sphagnum* mosses (Aerts et al., 2001; Gunnarsson and Rydin, 2000), a long-term elevated chronic Nr deposition could saturate the moss carpet layer and consequently leach downwards into the peat layer (Lamers et al., 2000; Gundale et al., 2011). The increased N availability could enhance peat decomposition rates and shift the microbial community status from N-limitation to P-limitation (Bragazza et al., 2004, 2012), whilst converting peatlands from C sinks to sources (Gunnarsson and Rydin, 2000). Consequently, *Sphagnum* mosses are outcompeted by vascular plants and their spatial coverage diminishes along with the associated BNF activity (Berendse et al., 2001; Wiedermann et al., 2007). Additionally, it has been reported that peatlands that are not any longer N or P limited could instead become K limited and change other macronutrients equilibria (e.g. Ca or Mg; Bragazza et al., 2004). Some metals such as Mo, Fe, and V also play a key role in regulating BNF (Bellenger et al., 2011; Warren et al., 2017).

Sulphur (S) emissions in the UK due to human activities peaked in the mid-seventies and have decreased ever since: wet S deposition has decreased about 70% and dry S deposition about 92%, between 1986 and 2008 (Mitchell et al., 2018), and now are, on average, below 3 kg S ha^{-1} yr^{-1}. Although S deposition in excess can have phytotoxic effects and can trigger

changes in grassland plant communities (Legge and Krupa, 2002; Mitchell et al., 2018), long-term elevated S deposition rates alone was found not to cause a swift in vegetation composition in a fertilization experiment in a peatland dominated by *Sphagnum* mosses (Wiedermann et al., 2007). However, no studies to date have evaluated the effects of elevated S deposition on BNF activity.

A factor that is essential for the nitrogenase enzyme to fix N_2 is the lack of oxygen (Postgate, 1982), which has been tied to moisture levels, this is that wet conditions can facilitate an anoxic environment (Chapin and Bledsoe, 1992). Several studies have found that an increase in moisture content resulted in an increase in BNF rates (e.g. Zielke et al., 2002; Rousk et al., 2018). Closely related, it has been reported that BNF increases with temperature up to an optimum degree that differs depending on the moss species (e.g. 16ºC for *S. riparium*, Basilier et al., 1978; between 20 and 30 ºC for *Pleurozium schreberi* and *Tomentypnum nitens*, Rousk et al., 2017). However, temperature and moisture have strong interactions with each other as high temperatures will dry mosses quickly (Rousk et al., 2015), thus their effects will depend greatly on the particular conditions of the peatland. Light is other factor that could affect BNF rates as some of the N_2-fixing bacteria rely on photosynthesis to get energy for the BNF process. Although Chapin and Bledsoe (1992) did not find a clear relationship, a study conducted at high altitude forests found that BNF rates decreased with light intensities over 500 µmol m^{-2} s^{-1} (Gundale et al., 2012); another study in arctic wetlands found that different light intensities (0-1000 µmol m^{-2} s^{-1}) had no effects on BNF rates in *Sphagnum* spp (Stewart et al., 2011a). Thus, there is no clear evidence about the effects of light on BNF activity. N_2-fixers are also sensitive to low pH. Basilier et al. (1978) found that BNF rates of *Sphagnum* mosses were higher with higher pH; and Waughman and Bellamy (1980) reported a similar trend for peat. pH in peatlands may be easily altered by Nr deposition and, therefore, it could affect bacterial community composition, metabolic

pathways, and BNF activity (Tang et al., 2019). Although the studies investigating these factors provided interesting information, it still cannot be fully understood how they affect BNF activity under natural variations in these conditions.

The analysis of the ^{15}N isotope in *Sphagnum* mosses provides information about the N sources they have used for growth (Zivkovic et al., 2017). If *Sphagnum* mosses take up N through BNF by bacteria their $\delta^{15}N$ signature would be close to zero, similar to the atmospheric N_2 (Stewart et al., 2011b). The type of atmospheric Nr deposition seems to play an important role in the $\delta^{15}N$ signature, so that elevated rates of ammonia emissions result in low values of $\delta^{15}N$ while elevated rates of NOx forms result in high $\delta^{15}N$ values (Bragazza et al., 2005; Zivkovic et al., 2017). On the other hand, if N is taken from the peat due to mineralization processes it will lead to a decrease in the $\delta^{15}N$ value (Knorr et al., 2015).

The main aims in this study were (1) to evaluate the effects of increased Nr deposition upon rates of BNF in peatlands; (2) to examine the main abiotic factors controlling BNF rates, particularly the effects of selected macro and micro nutrients on BNF; and (3) to examine if the suppression of BNF is associated with enhanced Nr uptake from Nr deposition in *Sphagnum* mosses and peat looking at their $\delta^{15}N$ signature.

3.3 Material and methods

3.3.1 Study sites

Samples were collected from four different peatlands which represent an atmospheric Nr deposition gradient. Three sites were in the United Kingdom: Fenn's and Whixall (52º 92' N 2º 72' W) in England, Migneint (52° 97' N - 3° 83' W) in Wales, and Forsinard (58° 38' N - 3° 92' W) in Scotland; and one, Degerö Stormyr (64° 11' N - 19° 33' E), located in

northern Sweden (Fig. 3.1). The latter site was selected as reference due to having low background Nr deposition rates. The four sites had different characteristics on precipitation, temperature, and Nr deposition (Table 3.1). The Nr deposition for each of the UK sites was obtained through the Air Pollution Information System (APIS) that used the Fine Resolution Atmospheric Multi-pollutant Exchange (FRAME) model to get a three years estimation (2014-2016) of the wet and dry N deposition (NH_4^+ and NO_3^-). Initially it was developed to model reduced nitrogen (Singles et al., 1998), but later adapted to incorporate oxidised nitrogen species (Fournier et al., 2004). The FRAME model is a Langrangian type one that simulates air columns moving along specific trajectories. While the emissions of ammonia were calculated using data from crops and fertilizer applications (agricultural emissions), non-agricultural emissions, and animal numbers (Dragosits et al., 1998), the emissions of NOx were obtained from the National Atmospheric Emissions Inventory for the UK (Salway et al., 1999). The N deposition data (wet and dry) was modelled at a grid resolution of 5 km x 5 km covering the period of study. The three years (2014-16) Nr deposition data for Degerö (Sweden) were obtained from the European Monitoring and Evaluation Programme (EMEP). This program started in 1977 for modelling Transboundary Long-Range Transported Air Pollutants in Europe covering a grid of 50 km x 50 km. The EMEP MSC-W model has changed extensively in the past ten years and it now covers different scales from local (about 5 km x 5 km grid) to more global (1-degree resolution). The model that was developed in the late seventies for sulphur compounds was shortly after further developed for nitrogen compounds (Iversen, 1990). The EMEP Eulerian model was unified in 2003 (Simpson, 2003), and for a full description of the EMEP MSC-W version see Simpson et al. (2012).

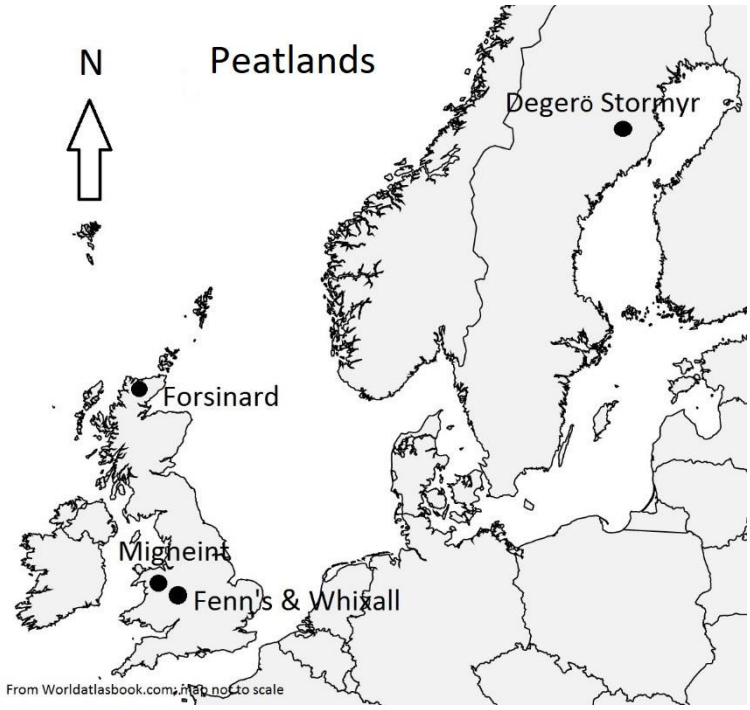

Figure 3.1. Locations of the sampling sites in the UK and Sweden.

3.3.2 Sampling campaigns

Two main sampling campaigns were carried out during the growing season in the study sites during which *in situ* incubations took place, one in 2016 and one in 2017, except in Forsinard and for the experimental fertilization treatment plots in Degerö Stormyr that were sampled only in 2017. Four dominant *Sphagnum* moss species as well as bulk peat (0 – 15 cm) from hollows and hummocks were collected for *in situ* incubations. Two species usually located in hollows (in pools or wet areas), *Sphagnum cuspidatum* and *S. fallax*, and two species that usually form hummocks (elevated and less wet areas), *S. capillifolium* and *S. papillosum*. In Degerö (Sweden) it was not possible to find the same exact species, except for *S. papillosum*, therefore similar ones were sampled (Atherton et al., 2010): in hollows *S. majus* and *S. balticum*; and in hummocks *S. fuscum* (Fig. 3.2).

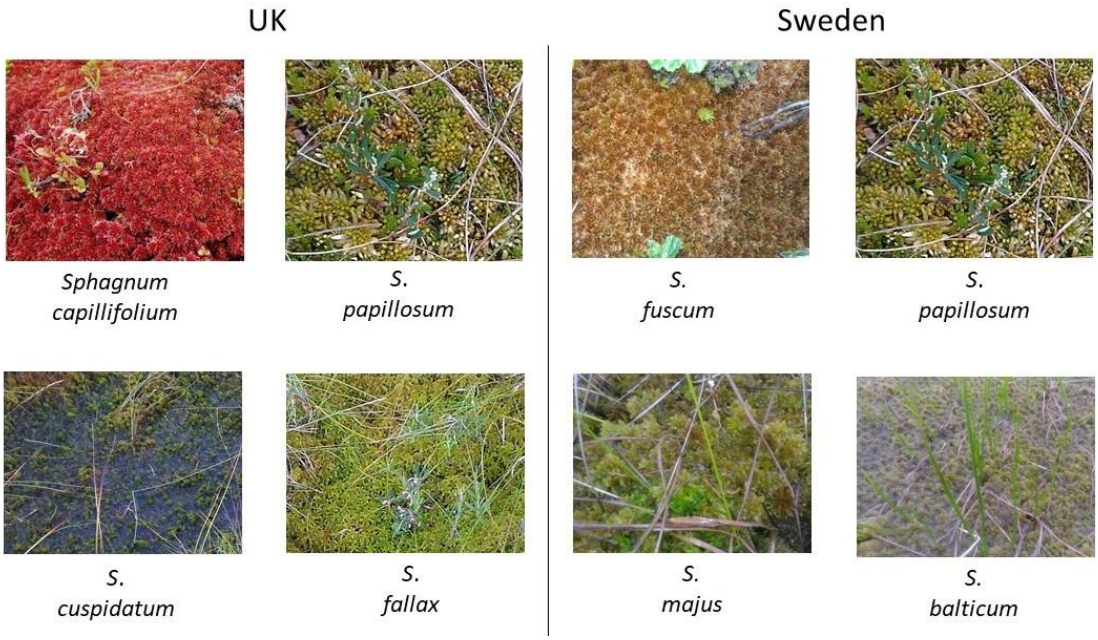

Figure 3.2. *Sphagnum* species collected in peatlands of the UK and Sweden from hummocks (upper row) and from hollows (bottom row).

Table 3.1. Mean annual temperature, precipitation and reactive nitrogen (Nr) deposition in the study sites.

Site	Mean annual temperature (°C)	Mean annual precipitation (mm)	Atmospheric Nr deposition (kg N ha^{-1} yr^{-1})	NH_4^+:NO_3^- ratio in atmospheric deposition
Degerö* (Sweden)	1.2	523	2	0.016
Forsinard (Scotland)	6.9	1104	6	0.026
Migneint (Wales)	7.3	2236	17	0.086
Fenn's and Whixall (England)	9.5	747	27	0.295

Source: Met Office (UK), Air Pollution Information System (APIS), European Monitoring and Evaluation Programme (EMEP), Eriksson et al., 2010b.

*MAT and MAP are the 30 years long-term average 1960-1991.

The incubation samples consisted of about 20 shoots (5 cm upper part) for each of the *Sphagnum* species, and about 10 grams of peat (homogenised through a 2 mm sieve) that were placed into 50 ml glass vials. After the insertion of the samples, the vials were closed with gas-tight rubber septa. At each sampling site, there were four incubation replicates and one control for each of the *Sphagnum* species and peat.

3.3.3 Degerö Stormyr treatment plots

At Degerö an experiment started in 1995 to evaluate the effects of increased air temperature combined with increased nitrogen and sulphur deposition. Plots (2 x 2 m) with two levels of each temperature (t), S, and N were established following a full factorial, giving a total of 21 plots including the control one. The treatment additions, one third after the snowmelt, and the rest every month from June to September in one-sixths doses of N were done as ammonium nitrate (NH_4NO_3) being the low level (n) of 15 Kg N ha^{-1} y^{-1} and the high level (N) of 30 Kg N ha^{-1} y^{-1}. For S the treatment additions were as sodium sulphate (Na_2SO_4) being the low level (s) 10 kg S ha^{-1} y^{-1} and the high level (S) 20 kg S ha^{-1} y^{-1}. No additions of N and S meant that water from the mire was used and the deposition was the natural background recorded for the area, 3 kg ha^{-1} y^{-1} for S and 2 kg ha^{-1} y^{-1} for N. The temperature was a qualitative variable being low or high that was raised by surrounding the plots with polycarbonate plates and covering them with a perforated film. Table 3.2 shows the description of the treatments for each plot. A detailed explanation of the experimental design and manipulations can be found in Granberg et al. (2001).

Table 3.2. Description of the treatments of the experimental plots at Degerö Stormyr.

Plot	Treatment	Description
0	None	Control of the experiment, no sampling (undisturbed).
1	n s	Low N and S: 15 and 10 kg ha^{-1} y^{-1} respectively.
2	N S t	High N and S: 30 and 20 kg ha^{-1} y^{-1} respectively, plus greenhouse.
3	S	High S: 20 kg ha^{-1} y^{-1}.
4	t	Greenhouse.
5	S t	High S: 20 kg ha^{-1} y^{-1}, plus greenhouse.
6	n s	Low N and S: 15 and 10 kg ha^{-1} y^{-1} respectively.
7	S	High S: 20 kg ha^{-1} y^{-1}.
8	N S	High N and S: 30 and 20 kg ha^{-1} y^{-1} respectively.
9	N t	High N: 30 kg ha^{-1} y^{-1}, plus greenhouse.
10	S t	High S: 20 kg ha^{-1} y^{-1}, plus greenhouse.
11	Control	No treatment, just mire water added.
12	N	Hig N: 30 kg ha^{-1} y^{-1}.
13	N S t	High N and S: 30 and 20 kg ha^{-1} y^{-1} respectively, plus greenhouse.
14	N S	High N and S: 30 and 20 kg ha^{-1} y^{-1} respectively.
15	n s	Low N and S: 15 and 10 kg ha^{-1} y^{-1} respectively.
16	t	Greenhouse.
17	N t	High N: 30 kg ha^{-1} y^{-1}, plus greenhouse.
18	N	High N: 30 kg ha^{-1} y^{-1}.
19	Control	No treatment, just mire water added.
20	n s	Low N and S: 15 and 10 kg ha^{-1} y^{-1} respectively.

3.3.4 Biological nitrogen fixation ($^{15}N_2$ direct assimilation method)

To measure BNF in the field the $^{15}N_2$ assimilation method was used as detailed in Saiz et al. (2019), so here it is explained just briefly. Immediately after the insertion of the samples in the vials, they were closed using rubber septa, 5 ml of air (10% of the headspace) was

replaced with $^{15}N_2$ gas (98 atom% Cambridge Isotope Laboratories Inc., USA). The gas was previously checked for contamination (Dabundo et al., 2014), and the data for BNF calculation corrected accordingly. Then the vials were placed upside-down (to avoid cap shade) in the same spot where the samples were collected (Fig. 3.3). In the case of the peat,

Figure 3.3. Field incubations of *S. cuspidatum* in Forsinard, Scotland.

they were located under the moss carpet. After 24 hours of incubation, the vials were opened and ventilated to flush out the remaining gas. They were placed into cool boxes with ice bags to transport them to the laboratory. Within the next three hours the samples were weighed (wet), dried at 70 ºC for 72 hours, and weighed again (dried) calculating, thus, the gravimetric moisture (% vol). Next, the samples were pulverised manually using a mortar, and subsamples were sent to Lancaster to the Life Sciences Mass Spectrometry Facility at the Centre for Ecology and Hydrology, to be analysed for ^{15}N content in peat and tissues by Isotope Ratio Mass Spectrometry (IRMS) using an elemental analyser Carlo Erba NA1500 (Italy) coupled to an isotope ratio mass spectrometer Dennis Leigh Technologies (UK). The

analytical precision of the instrument was 0.36 ‰. The analysis of all the samples (control and enriched) was done in duplicate (Jardine and Cuniak, 2005) being the variability within the analytical precision. To calculate the BNF rates the following formula was used (Equation 3-2; Liengen, 1999):

$$Y = \left(\frac{atom\%\ ^{15}Nexcess}{100}\right) x \left(\frac{totalNsample\ x\ 10^9}{t\ x\ 28}\right) x\ \left(\frac{100}{\%^{15}N\ air}\right)$$

where Y (nmol N gdw^{-1} h^{-1}) is the amount of N_2 fixed during the experiment, atom% $^{15}Nexcess$ is the difference between $atom\%^{15}Nsample$ and $atom\%^{15}Ncontrol$, total N is the total amount of nitrogen in the sample (g N 100 gdw^{-1}), t is the incubation time, 28 is the molecular weight of N_2 (g/mol), and $\%^{15}Nair$ is the percentage of ^{15}N out of the total amount of N gas in each incubation vial.

3.3.5 Calculation of growing season nitrogen inputs from BNF measurements

The nitrogen loads of the system were calculated by surface area from the BNF rates. To do so, it was used the average surface area of the different *Sphagnum* species and peat that was occupied by the shoots (or 10 grams of peat) that were inserted into the vials. This way was possible to change the mass units into surface units. So through conversions, the units could go from nmol N gDW^{-1} d^{-1} to kg N ha^{-1} yr^{-1}. Then it was calculated the percentage of coverage of the *Sphagnum* species with regards to other species and vascular plants. The digital image analysis technique (Baxendale et al., 2016) was used to determine the percentage of coverage of the *Sphagnum* mosses. The analysis of the results suggested that the percentage of coverage by *Sphagnum* mosses were 50%, 45%, 55%, and 70% for Fenn's

and Whixall, Migneint, Forsinard, and Degerö respectively. In order to get a more accurate approximation of the nitrogen inputs into the system it was considered the growing season that is the period of the year in which the daily mean temperature (five days in a row) is over +5 ºC (Eriksson et al., 2010a). The growing season for Fenn's & Whixall was of 280 days, for Migneint of 255 days, for Forsinard of 200 days, and for Degerö of 150 days.

3.3.6 Elemental analyses in *Sphagnum* tissue and peat

A set of subsamples of pulverized *Sphagnum* tissue and peat from each of the species of each site were analysed for total C and N content. They were sent to the laboratory of the School of Geographical Sciences of the University of Bristol. They were analysed using a Thermo Scientific FlashEA 1112 Nitrogen and Carbon analyser. The instrument had a limit of detection (LOD) for both C and N of 0.01%, and the precision was determined by repeated analysis of a soil reference standard (0.21% N and 2.39% C) and the relative standard deviation (RSD) was below 5%.

Of the ground samples 0.2 grams were digested in 9 ml of HNO_3 (>68%) trace metal grade and 1 ml of H_2O_2 (30%) ACS grade using a microwave Mars 6 CEM (Mathews, NC, USA). Then the digests were diluted using deionised water and analysed for total P and metals (Mg, K, Ca, V, Mn, Co, Ni, Cu, Mo) using inductively coupled plasma – mass spectrometry (ICP-MS, Perkin Elmer NexION 300D, Waltham, MA, USA). An 8-point calibration generated through dilution of a certified multi-component standard (VWR, UK) was used to determine the values. Additionally, every 9 samples a blank and a quality control samples were included. The results were blank corrected. On average, the RSD was below 4% for all the elements while the LOD was 0.3 μg/g for Mg, K, Ca, Ni, Mo, and P; and 0.1 μg/g for V, Mn, Co, and Cu.

3.3.7 Ancillary measurements in the field

After each incubation in the field it was recorded the mean ambient temperature (ºC) for the sites on that date from stations nearby and measured the temperature in the moss and peat (5-10 cm depth) at the exact location where the samples were taken and incubated using a stem thermometer probe (Premier Farnell Ltd, UK), factory calibrated, with a manufacturer-specify accuracy of ±1 ºC and a resolution of 0.1 ºC. Additionally, at the exact same spots it was measured photosynthetically active radiation (PAR, µmol 100 m^2 s^{-1}) using a portable light meter (LI-250A, LI-COR, Linclon, Nebraska, USA) with a quantum sensor that was provided with the calibration multiplayers that were entered when it was installed, and the manufacturer-specified accuracy was of ±0.6% of reading. Dissolved oxygen (DO, mg/l) was also measured using a portable DO meter (HACH HQ40d with LDO probe, Loveland, CO, USA) that was calibrated daily using the water-saturated air (100%) calibration procedure and that had a detection limit of 0.05 mg/L and an accuracy of ±0.1 mg/L indicated by the manufacturer. pH using a pH meter (HI-98100 Hanna Instruments, Leighton Buzzard, UK) that was calibrated daily using certified pH 7 and pH 4 buffer solutions (Fisher Scientific, UK) and that had an accuracy of ±0.2 pH. Electrical conductivity (EC, µS/cm) using an EC meter (HI-98300 Hanna Instruments, Leighton Buzzard, UK) that had the temperature automatically adjusted, was calibrated daily using a certified 1413 µS cm^{-1} stock solution (VWR, UK), and had an accuracy of ±2% F.S. and a resolution of 1 µS cm^{-1}. And also soil moisture focused on capturing the moisture of the moss carpet (5-10 cm upper part) and peat layer (5-10 cm depth from the beginning of peat in hollows and hummocks) using a moisture meter type HH2 (Delta-T Devices, Burwell, UK) that was calibrated using the sensor calibration file provided by the manufacturer, and had a resolution of 1 mV (range from 0 to 1500 mV) and an accuracy of ±(0.13 of mV reading + 1 mV).

Pore water samples were also taken from the incubation locations at each site. The samples were transported to the laboratory immediately after collection in a cool box. Then they were filtered through a Restek 0.45µm PTFE syringe filter, 25mm diameter. Following, they were analysed for nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulphate (SO_4^{2-}) concentration using an ion chromatograph (DIONEX ICS-1000, Sunnyvale, CA, USA). A 6-points calibration curve was generated from serial dilutions of a certified stock solution (Sigma-Aldrich, UK) to determine the unknown samples. Every six samples a quality control and a laboratory blank were included. The limits of detection were 0.1 mg L^{-1} for NO_3^-, <0.001 mg L^{-1} for $PO^3_4^-$, and 0.2 mg L^{-1} for SO_4^-. The results were blank corrected and the precision as RSD was <5%. Also, the samples were analysed for ammonia (NH_3) using a flow injector analyser (Lachat QuikChem 8500, Hach, Loveland, CO, USA). To determine the values a 9-points calibration curve was generated from serial dilution of a certified stock solution (Sigma-Aldrich, UK). A laboratory blank and a quality control samples were included every six samples, the limit of detection for NH_3 was 0.07 mg N L^{-1}, the results were blank corrected, and the RSD was <5%.

Peat samples from hollows and hummocks (10 g) were extracted with 50 ml of deionised water and 50 ml of 2 M KCl for the determination of nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulphate (SO_4^{2-}); and ammonia (NH_3) as shown in Fig. 3.4. Briefly, the peat slurries were shaken in an automatic shaker for 1 hour at 400 rpm, and after they were centrifuged at 2849 x g rcf (4200 rpm) for 30 minutes followed by a double filtration in which every nine samples a blank (deionised water) was included, first through a number 42 Whatman filter paper, and second through a 0.45 µm PTFE Restek 25 mm diameter syringe filter. The analysis was performed as indicated above for the pore water.

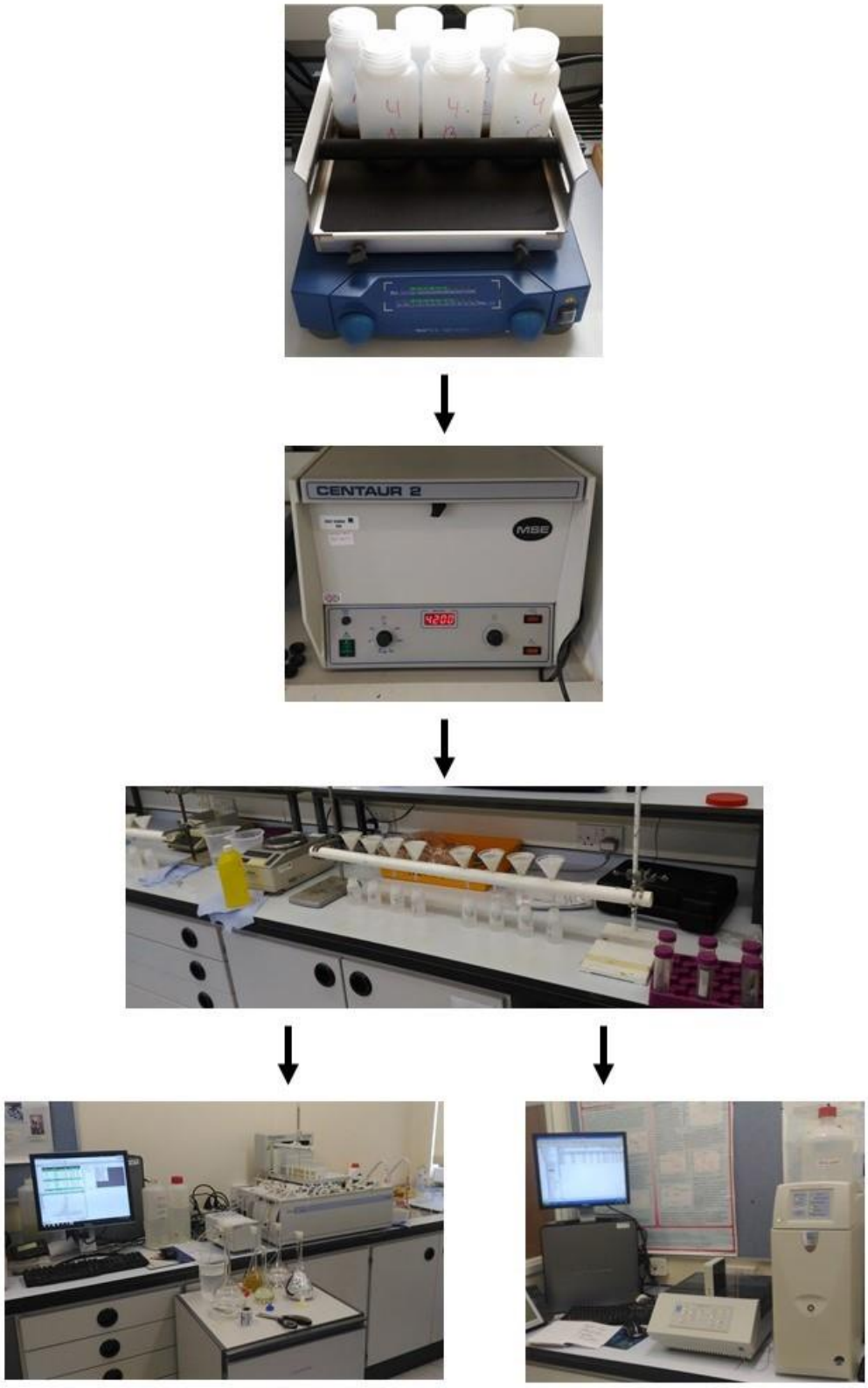

Figure 3.4. A flowchart showing the peat extraction process for analysis once the peat samples were mixed in a 1:5 ratio with deionised water in Nalgene bottles: first the samples were shaken, then centrifuged, filtered, and finally analysed using a flow injector analyser (left) or an ion chromatograph (right).

3.3.8 Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics software, version 24. It was tested the data for normality (Shapiro-Wilk) and for homogeneity of variance (Levene's test). The statistical analysis was done using non-parametric tests when non-normal and non homogeneous data was found. To test correlations between two variables among paired samples it was used the Spearman's rank-order correlation. The bootstrapped t-test was used to look for differences in paired samples. The differences by site and the differences by species or by treatments in the same site were measured using the Kruskal-Wallis test, followed by pairwise comparisons. Significant differences were considered at $P < 0.05$.

3.4 Results

3.4.1 BNF across an Nr deposition gradient

The results from two growing seasons (2016-2017) show that there was a significant difference among sites ($P < 0.01$) regarding median BNF rates (Fig. 3.5). Also, a significant inverse correlation between BNF and Nr deposition ($P < 0.001$) was observed when considering the overall data (2016 and 2017; Fig. 3.5). This relationship was also found between the median BNF rates in 2016 and in 2017 and Nr deposition (data not shown). The median BNF rates measured across sites (in 2016, 2017, and overall 2016-17) decreased as Nr increased. In addition, regarding the Nr composition (NH_4^+ and NO_3^-) it was observed a significant ($P < 0.01$) negative correlation between NH_4^+:NO_3^- ratio and BNF rates among sites, i.e. the higher the ratio the lower the BNF rates.

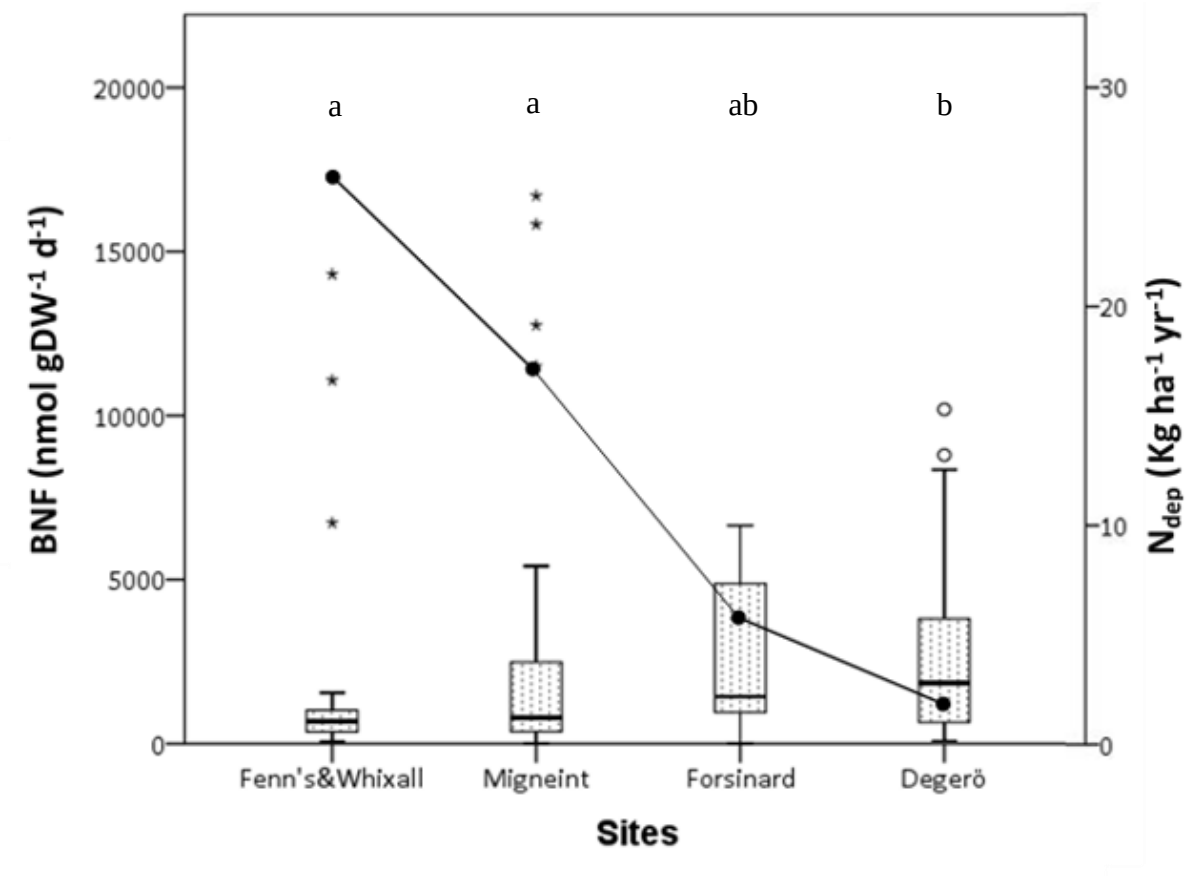

Figure 3.5. Boxplot of the data (2016-2017) of BNF rates (left y-axis; nmol gDW^{-1} d^{-1}) at each sampling site: Migneint, Fenn's and Whixall, Forsinard and Degerö (n=39 except Forsinard n=15). And N deposition (right y-axis; kg ha^{-1} yr^{-1}) at each sampling site (black dot connected by straight line). The box shows the median (central line), the 25^{th} (lower part) and 75^{th} (upper part) percentiles with whiskers indicating the minimum and maximum values. The white dots show outliers (1.5-3 IQR) and the stars extreme values (>3 IQR). Sites with different letters are significantly different. Kruskal Wallis Test: H(3) = 11.499, P = 0.009, with mean rank of 56.96 for Fenn's&Whixall, of 63.49 for Migneint, of 84.00 for Forsinard, and of 84.81 for Degerö.

High levels of Nr deposition > 10 Kg N ha^{-1} yr^{-1}, which is the threshold, i.e. the critical load, over which it has been reported that BNF shuts down in the UK (Dore et al., 2012) and in northern ecosystems (Rousk and Michelsen, 2016) partially suppressed BNF, but did not shut it down. The suppression ratio obtained (rate of BNF reduced, nmol of N gDW^{-1} d^{-1}, per unit of Nr deposition, in Kg N ha^{-1} yr^{-1}), using Degerö as reference, is 9.3 times higher in Forsinard than in Migneint (in 2017), and 1.4 times higher in Migneint than in Fenn's and

Whixall (in 2016 and in 2017), thus it was observed that the suppression effect of Nr deposition on BNF was higher in areas where the Nr deposition was lower and decreased as Nr deposition increased. In short, that more Nr deposition reduced the suppression effects on BNF.

Regarding BNF rates per species, the results showed a significant difference among species (P = 0.006). The main significant differences were between mosses (Degerö species included; median of 1119.4 ± median absolute deviation-MAD of 822.6 nmol N gDW^{-1} d^{-1}) and peat (median of 684.0 ±MAD of 279.4 nmol N gDW^{-1} d^{-1}), and within the *Sphagnum* mosses the one that stood out over the rest was one of the species in hollows: *S. fallax* (1660.2 ±MAD 1355.9 nmol N gDW^{-1} d^{-1}) (Fig. 3.6). The results also showed that *Sphagnum* species in hollows (*S. cuspidatum* & *S. fallax*) fixed 68.8% more (median of 1499.7 ±MAD 1339.9 nmol N gDW^{-1} d^{-1}) than the ones in hummocks (*S. capillifolium* & *S. papillosum*; median of 888.4 ±MAD 607.4 nmol N gDW^{-1} d^{-1}) considering the all the data (2016-2017), including Degerö speices.

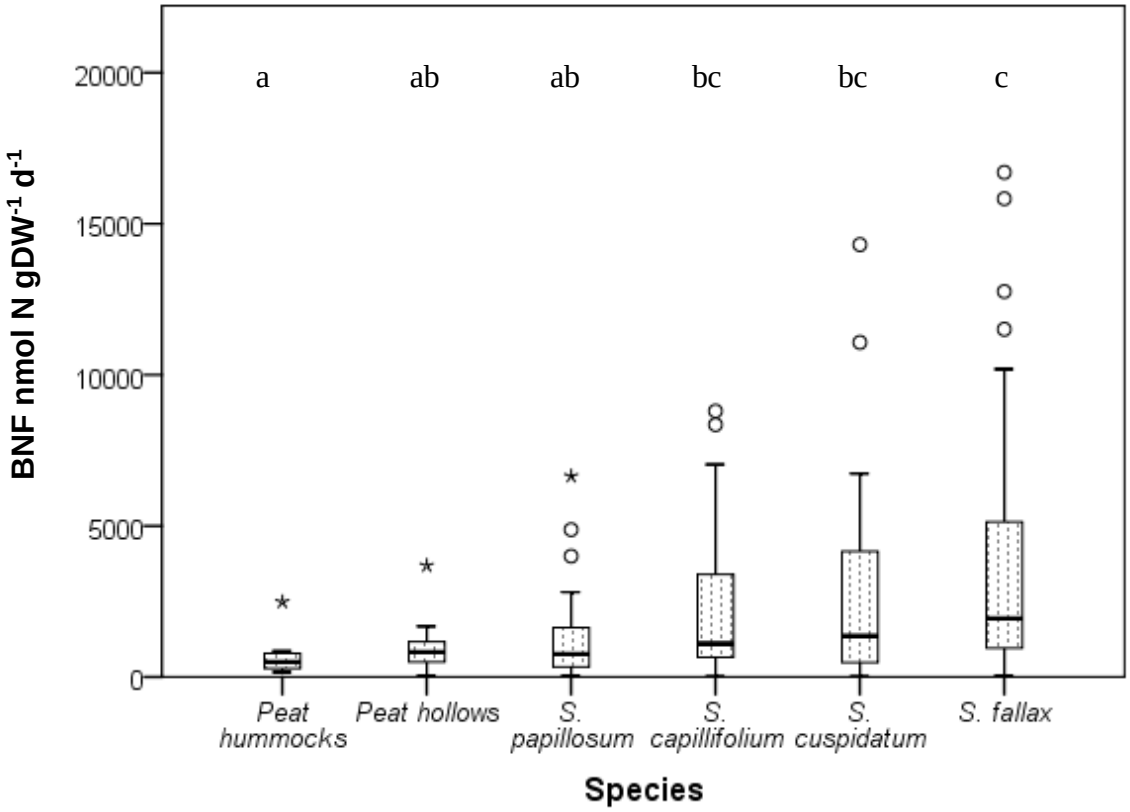

Figure 3.6. Boxplot of the overall (2016-2017) BNF rates (nmol gDW^{-1} d^{-1}) per *Sphagnum* moss species and peat (n=27). Also included Degerö *Sphagnum* species *S. fuscum* in *S. capillifolium*, *S. majus* in *S. cuspidatum*, and *S. balticum* in *S. fallax*. The box shows the median (central line), the 25^{th} (lower part) and 75^{th} (upper part) percentiles with whiskers indicating the minimum and maximum values. The dots show outliers (1.5-3 IQR) and the starts extreme values (>3 IQR). Kruskal Wallis Test: H (5) = 16.295, P = 0.006. Species with different letters are significantly different.

The loading rates of N to the system have an inverse relationship with the Nr deposition gradient despite the differences in the length of the growing season and the percentage of moss coverage (Table 3.3).

Table 3.3. Average BNF (Kg N ha^{-1} yr^{-1}), percentage of surface covered by *Sphagnum* species, the number of days considered growing season at each site, and the resulted N load (Kg N ha^{-1}) per growing season (gs). The BNF and N load data shown are medians (±median absolute deviation-MAD). Sites with different letters (N load column) are significantly different.

Sites	BNF (Kg ha^{-1} yr^{-1})	*Sphagnum* cover (%)	Growing season (Days)	N load (Kg N ha^{-1} gs^{-1})
Fenn's&Whixall	11.9 (±6.7)	50	280	4.56 (±2.57) a
Migneint	17.6 (±15)	45	255	5.55 (±4.71) a
Fordsinard	24.8 (±24.5)	55	200	7.47(±7.37) ab
Degerö	56.7 (±36.4)	70	150	16.32 (±11.05) b

3.4.2 Environmental factors affecting BNF

Table 3.4 shows the medians of selected environmental variables. A significant (P = 0.042) positive correlation between BNF rates and nitrate pore water concentration was found. In contrast, it was found a negative significant correlation (P = 0.029) with ammonia in peat (Table 3.4). No other significant correlations between BNF and these variables (in Table 3.4) were found.

Table 3.5 shows the median concentration of the selected elements for *Sphagnum* mosses and peat. It was found a significant positive correlation between BNF rates and calcium (P = 0.039) and a negative one with manganese (P = 0.004). No other significant correlations were found between BNF and these selected elements (in Table 3.5). In addition, it was found a significant (P < 0.01) positive correlation between Nr deposition and the concentration of Ni, Cu, Mo, and P at each site, and also a negative one with the C:P ratio.

Table 3.4. Selected environmental, pore water and peat properties. Data shown is median (±MAD) per site for years 2016 and 2017 (except Forsinard: only 2017) (n=36 except for peat data n=12. Forsinard half these values). Sites with different letters are significantly different.

Table		**Fenn's & Whixall**	**Migneint**	**Forsinard**	**Degerö**
Abiotic factors	**Units**	Median (±MAD)	Median (±MAD)	Median (±MAD)	Median (±MAD)
Moss/peat Temperature	°C	**12.4** (±0.9) a	**12.9** (±1.1) b	**10.2** (±0.2) c	**17.1** (±2.4) d
Photosynthetic Active Radiation (PAR)	µmol*100* $m^{-2}*s^{-1}$	**336.9** (±88.9) a	**587.3** (±334.8) a	**146.7** (±12.3) b	**3.6** (±0.6) c
Dissolved Oxygen	mg/L	**4.1** (±2.3) a	**5.7** (±1.0) a	**7.0** (±0.4) b	**ND**
pH		**3.8** (±0.2) a	**4.6** (±0.1) b	**5.0** (±0.2) c	**3.8** (±0.1) a
Electrical Conductivity	µS/cm	**94.5** (±10.0) a	**30.0** (±9.0) b	**38.0** (±1.5) c	**45.0** (±11.0) b
Sphagnum/Peat moisture	% vol	**65.0** (±25.6) a	**46.9** (±34.4) a	**76.9** (±16.1) a	**64.8** (±33.7) a
Gravimetric moisture	g/g	**20.7** (±8.7) a	**17.4** (±4.8) a	**17.4** (±6.82) a	**16.0** (±7.9) a
Pore Water NO_3	mg/L	**0.256** (±0.002) a	**0.101** (±0.008) b	**0.167** (±0.011) a	**0.112** (±0.008) b
Pore Water NH_3	mg/L	**0.114** (±0.024) ac	**0.092** (±0.021) a	**0.059** (±0.005) c	**0.123** (±0.038) c
Pore Water PO_4	mg/L	**0.279** (±0.050) a	**0.333** (±0.102) a	**0.328** (±0.004) a	**0.242** (±0.013) a

Pore Water SO_4	mg/L	**0.151** (±0.030) a	**0.453** (±0.081) b	**0.537** (±0.084) b	**0.135** (±0.058) a
Peat NO_3	µg/g	**1.200** (±NA) a	**1.013** (±0.389) a	**1.003** (±0.01) a	**1.280** (±0.051) a
Peat NH_3	µg/g	**3.865** (±1.748) a	**6.133** (±5.851) a	**0.090** (±0.044) b	**2.310** (±1.820) a
Peat PO_4	µg/g	**1.735** (±0.632) a	**10.366** (±0.639) b	**5.002** (±0.513) bc	**3.119** (±0.907) ac
Peat SO_4	µg/g	**4.881** (±3.831) ab	**3.876** (±1.463) a	**2.268** (±0.471) b	**1.669** (±1.039) b

Table 3.5. Elements in *Sphagnum* tissue and peat. Data shown is median (±MAD) per site (n=18) for 2017. Sites with different letters are significantly different.

	Fenn's&Whixall	Migneint	Forsinard	Degerö
Elements	µg/g (±MAD)	µg/g (±MAD)	µg/g (±MAD)	µg/g (±MAD)
Mg	**488.10** (±107.53) a	**985.30** (±387.54) b	**1224.82** (±102.38) c	**383.20** (±103.09) a
K	**4283.18** (±1034.1) a	**2148.83** (±1120.3) b	**1853.81** (±1128.6) b	**2548.74** (±719.21) b
Ca	**367.15** (±153.83) a	**216.82** (±198.75) a	**403.28** (±205.51) a	**272.12** (±181.87) a
V	**79.43** (±1.83) a	**78.88** (±1.39) b	**71.65** (±3.21) c	**74.27** (±0.87) bc
Mn	**83.25** (±18.08) a	**79.80** (±30.94) a	**31.83** (±4.32) a	**41.47** (±9.29) a
Co	**19.55** (±1.41) a	**19.93** (±1.89) ab	**16.56** (±1.28) b	**13.16** (±0.12) c
Ni	**296.13** (±7.70) a	**292.46** (±4.87) b	**246.67** (±1.61) bc	**243.85** (±2.26) c
Cu	**56.49** (±2.03) a	**56.17** (±32.40) a	**50.43** (±3.64) a	**16.69** (±10.94) a
Mo	**332.45** (±32.26) a	**329.43** (±5.97) b	**286.88** (±4.58) b	**285.95** (±3.02) b
	mg/g (±MAD)	mg/g (±MAD)	mg/g (±MAD)	mg/g (±MAD)
C	**448.55** (±7.76) a	**437.92** (±6.34) a	**446.20** (±7.29) a	**445.33** (±5.85) a
N	**6.22** (±1.29) a	**5.88** (±0.73) a	**7.34** (±2.23) a	**5.36** (±0.94) a
P	**0.41** (±0.04) a	**0.35** (±0.08) a	**0.33** (±0.10) a	**0.30** (±0.04) a
Ratios				
C:N	**72** (±18) a	**73** (±12) a	**59** (±25) a	**82** (±20) a
C:P	**1097** (±123) a	**1249** (±276) a	**1330** (±523) a	**1460** (±175) a
N:P	**12** (±1) a	**17** (±4) a	**21** (±6) a	**17** (±3) a

3.4.3 Variability in $\delta^{15}N$ signature

The $\delta^{15}N$ values decreased with the increase of Nr deposition in the UK from a median value of -1.49 ‰ in Forsinard with a rate of Nr deposition of 6 kg N ha^{-1} y^{-1}, to -5.73 ‰ in Fenn's & Whixall with 27 kg N ha^{-1} y^{-1} (Fig. 3.7), and it was found a significant correlation ($P < 0.01$) between Nr deposition and $\delta^{15}N$. The median $\delta^{15}N$ value found in Degerö was -2.26

‰, a little bit lower than the one of Forsinard. Regarding the NH_4^+:NO_x ratio (Table 3.1) for the study sites, it was found a significant correlation ($P < 0.05$) with the $\delta^{15}N$ signature, as the ratio decreased (F&Whixall>Migneint>Forsinard>Degerö), the $\delta^{15}N$ values increased.

The *Sphagnum* species forming hummocks, *S. capillifolium* (including *S. fuscum*), and *S. papillosum* had a median $\delta^{15}N$ value of -4.72 ‰ and -4.18 ‰ respectively which were the lowest. The median $\delta^{15}N$ signature for the species in hollows *S. cuspidatum* (including *S. majus*) and *S. fallax* (including *S. balticum*) was of -2.63 ‰ and -2.92 ‰ correspondingly. The peat from hollows and from hummocks had values closer to 0: -0.08 ‰ and -0.585 ‰ respectively (Fig. 3.8).

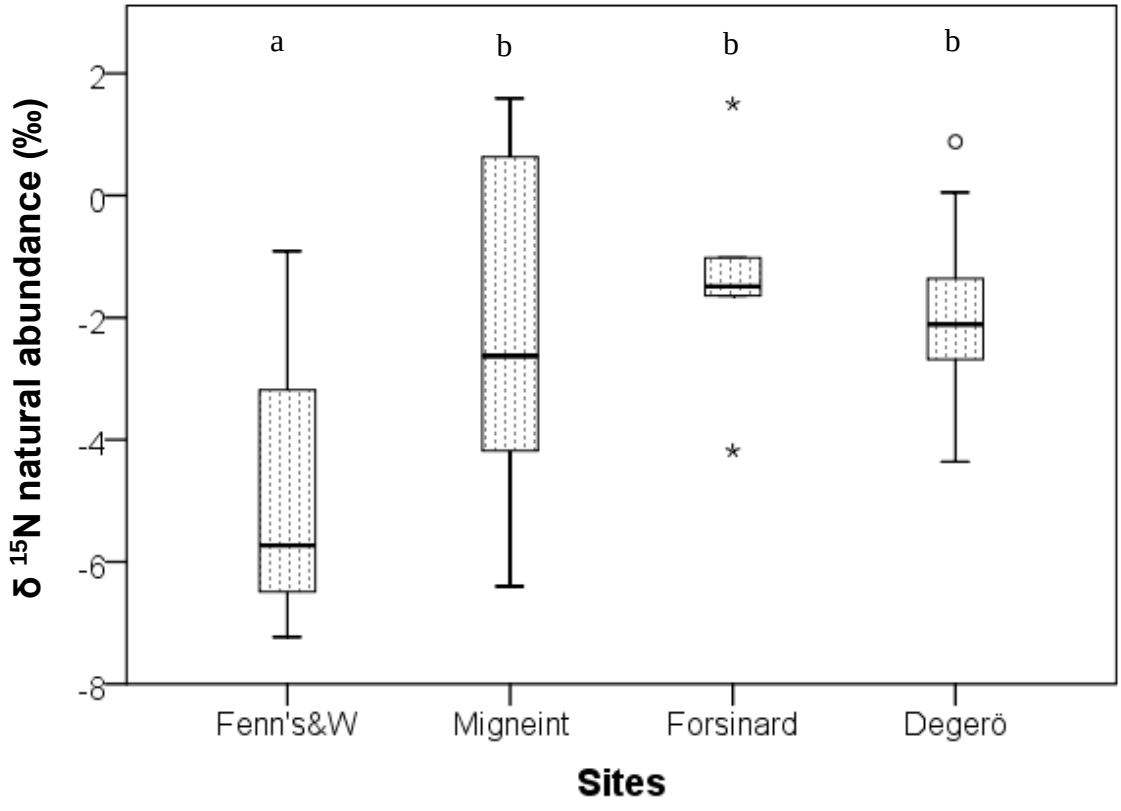

Figure 3.7. Boxplot of the $\delta^{15}N$ natural abundance in ‰ (n=12 except Forsinard n=6) at the four sampling sites. The box shows the median (central line), the 25th (lower part) and 75th (upper part) percentiles with whiskers indicating the minimum and maximum values. The dots show outliers (1.5-3 IQR) and the starts extreme values (>3 IQR).

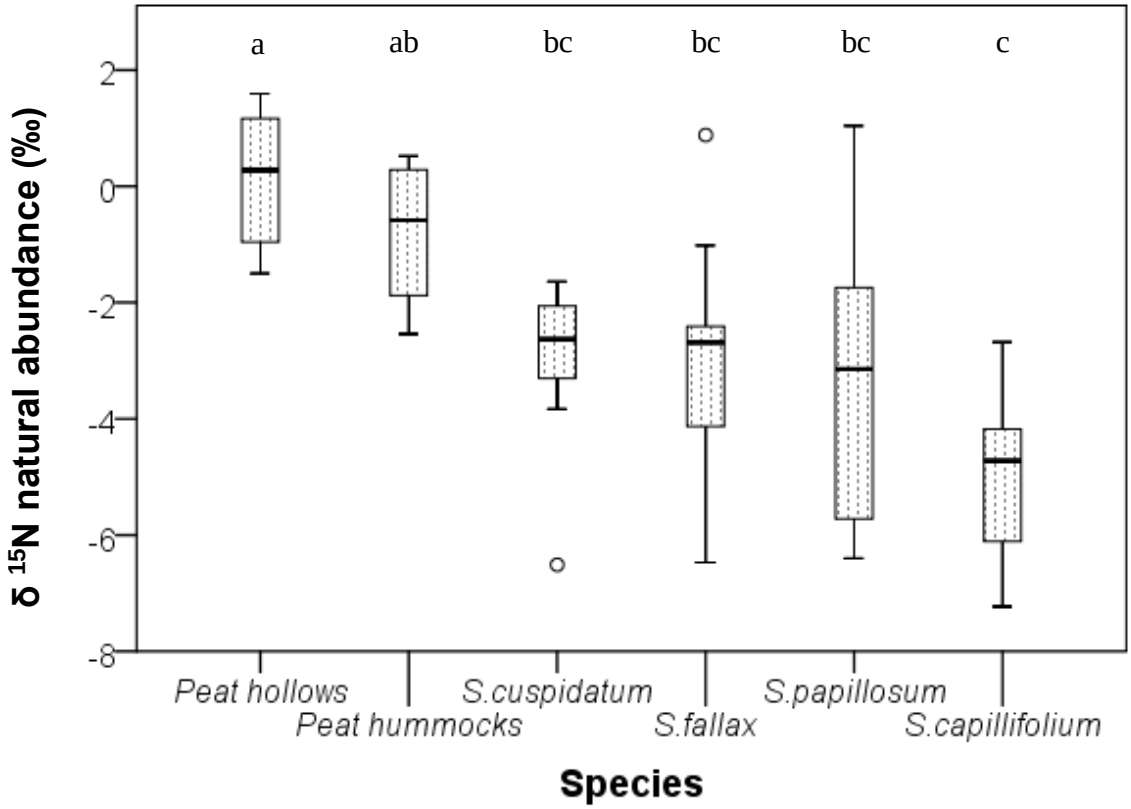

Figure 3.8. Boxplot of the $\delta^{15}N$ natural abundance in ‰ (n=7) of the six different species of *Sphagnum* and peat studied (including the Degerö species, see Fig. 3.5). The box shows the median (central line), the 25th (lower part) and 75th (upper part) percentiles with whiskers indicating the minimum and maximum values. The dots show outliers (>1.5 IQR).

3.4.4 Degerö treatment plots

The results of the Degerö treatment plots incubations (Fig. 3.9) show that after more than two decades of nitrogen, sulphur, and temperature treatments, BNF did not shut down although it was reduced. The treatments with a significant reduction in comparison to the control plots (median of 3126.26 nmol N gDW^{-1} d^{-1}) were ns, SN T, NS, and N, with median

rates of 393.05, 200.99, 331.46, and 1122.63 nmol N gDW^{-1} d^{-1} respectively. Other treatments resulted also in a considerable decrease such as T with a rate of 877.17, S T of 887.22, and N T of 1013.76 nmol N gDW^{-1} d^{-1}. In addition, regarding S, although the BNF rates were overall lower than the control ones, in one of the two plots with the treatment, the rates were higher than the median of the control plots.

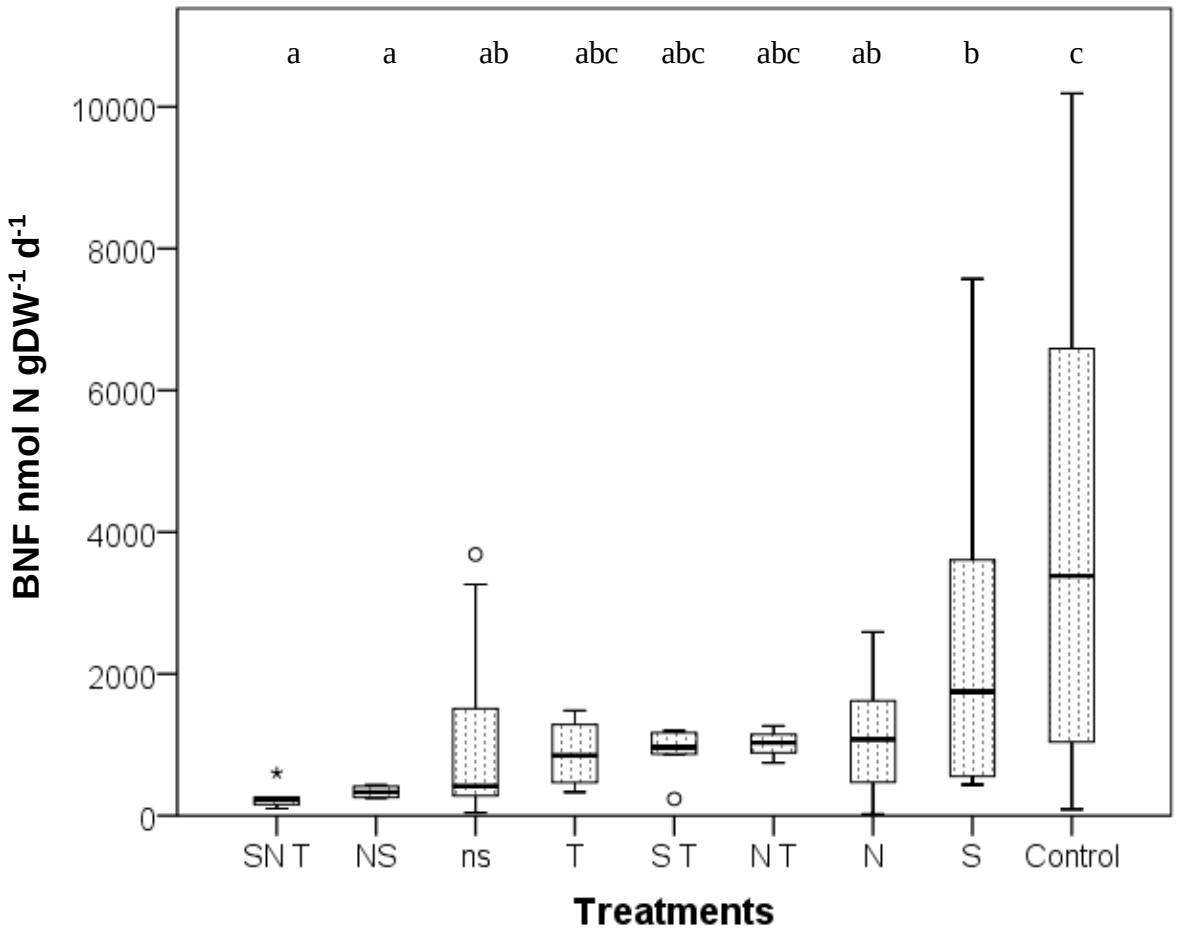

Figure 3.9. BNF rates (nmol N gDW^{-1} d^{-1}) of the *Sphagnum* spp under different treatments (n=4). The box shows the median (central line), the 25th (lower part) and 75th (upper part) percentiles with whiskers indicating the minimum and maximum values. The dots show outliers (1.5-3 IQR) and the starts extreme values (>3 IQR). Sites with different letters are significantly different. (S, sulphur; N, nitrogen; T, temperature; and the combined treatments).

3.5 Discussion

In this study, it was found that BNF rates decreased as Nr deposition rates increased, however, BNF activity did not entirely shut down (Fig. 3.5). These results agree with those of other studies on feather mosses in boreal forests in different locations following an Nr deposition gradient (Zackrisson et al., 2009; Leppänen et al., 2013; Rousk et al., 2013). They are also consistent with laboratory studies on Nr addition effects using *Sphagnum* mosses collected from peatlands (Kox et al., 2016), or with field studies, in feather moss carpets of a boreal forest, but using experimental plots with additions of different concentrations of Nr (Gundale et al., 2011). The suppression effect of Nr deposition on BNF was higher (per unit of Nr deposition) in areas with lower Nr deposition rates than in areas with high Nr deposition rates and it decreased as Nr deposition increased. This fact may explain the results reported by Zackrisson et al. (2004) that showed almost total inhibition of BNF after N additions of 4.25 kg N ha^{-1} yr^{-1} in feather mosses with an Nr deposition background of <0.5 kg N ha^{-1} yr^{-1}; and also the findings by van den Elzen et al. (2018) that showed no effects after eleven years (2002-2013) of Nr additions of 24 kg N ha^{-1} yr^{-1} on BNF rates of different moss and lichen species of a peatland, Whim bog in Scotland, with Nr background of 8 kg N ha^{-1} yr^{-1}. Based on these background rates, the Centre for Ecology & Hydrology calculated the total N deposition data for three years (2011-2013) using the modelling tool CBED (Concentration Based Estimated Deposition) giving an average value for the Whim bog area of 14.9 kg N ha^{-1} yr^{-1}, which is higher than the mentioned in the study done in 2013 (van den Elezen et al., 2018). Other studies have also indicated that high rates of Nr deposition, up to 50 kg N ha^{-1} yr^{-1}, did not shut down BNF in tropical forests (Zheng et al., 2018), nor in heavy polluted forested peatlands (Zechmeister-Boltenstern and Kinzel, 1990). This gradual increase over decades in the Nr deposition rates above the natural background may have affected the N_2-fixers population making them less sensitive to high rates of Nr deposition.

In fact, this study found a similar percentage of suppression in the median BNF rates for 2016 and 2017 in Fenn's and Whixall (63%) compared to the treatment plots in Degerö after more than 20 years of Nr addition at the rate of 30 kg N ha^{-1} yr^{-1} (64%) which is close to the Nr deposition of the former. Thus, the results suggest that it is important not to disregard BNF activity in areas with high Nr deposition rates.

The BNF rates found in northern Sweden (median of 3126 nmol N gDW^{-1} d^{-1}) were in accordance with those of other studies in similar pristine peatlands from Sweden (320-4880 nmol N gDW^{-1} d^{-1}; Basilier et al., 1978) or Finland (up to 3024 nmol gDW^{-1} d^{-1}; Larmola et al., 2014). In Fenn´s and Whixall with a Nr deposition of ~27 kg N ha^{-1} yr^{-1} and Migneint with ~17 kg N ha^{-1} yr^{-1} the median of BNF that was found was 826.2 and 983.1 nmol N gDW^{-1} d^{-1} respectively which is in the range of that reported by van den Elzen et al. (2017) of 960 nmol N gDW^{-1} d^{-1} in an experiment with *Sphagnum* mosses from a peatland in the Netherlands with a Nr deposition rate of 25 kg N ha^{-1} yr^{-1}. These results, however, suggest far higher rates than those reported by Zechmeister-Boltenstern and Kinzel (1990) for Austria (Central Europe), region that suffered from heavy pollution during most of the twentieth century (1948-1989; Puxbaum and Gregori, 1998; Novak et al., 2014). This pollution may be the reason for this difference because, as it was got in the experimental plots in Degerö (Fig. 3.9), the combination of high rates of Nr and S deposition reduced BNF rates up to ~90%.

Considering the different *Sphagnum* species, it was found that the ones from hollows had higher BNF rates (almost 70% more) than the ones forming hummocks. Following the same pattern, the bacterial community from peat in hollows fixed 67% more N_2 than the one from peat in hummocks. These results are in agreement with those of other studies that have measured BNF rates in flarks and hummocks of fens (Larmola et al., 2014), in hollows and hummocks of biological soil crusts (Stewart et al., 2011b), or in hollows and hummocks of

a bog located in an experimental forest (Warren et al., 2017). It was discovered that *Sphagnum* mosses fixed almost 64% more N_2 than peat which is within the range recorded by Stewart et al. (2011a) between *Sphagnum* spp and soil. Thus, it is important to sample all peatlands habitats in order to get a more accurate estimation of landscape-scale BNF.

Four significant correlations were found between BNF and the studied variables. In *Sphagnum* tissue and peat it was detected a significant positive correlation between BNF and calcium, contrarily to what was reported by Waughman and Bellamy (1980), and a negative one with manganese showing that the less content of this heavy metal the higher the BNF rates. It was also found a significant positive correlation between BNF and nitrate in pore water, which could be because this form (NO_3^-) is not as readily available as NH_4^+, and it can be converted to different N forms (van den Elzen et al., 2018). A significant negative correlation between BNF and ammonia in peat was discovered, which is consistent with the fact that *Sphagnum* mosses act as filters of Nr deposition, specifically for ammonium that is taken 8 times faster than nitrate (Fritz et al., 2014), and in excess it is toxic for mosses. Thus, if the deposition rates exceed a certain threshold leaching to the peat layers below may occur (Bragazza and Limpens, 2004; van den Elzen et al., 2018). This finding highlights the potentially severe impact of ammonium fertilization of agricultural fields on BNF of natural ecosystems.

The median $\delta^{15}N$ values found in the UK sites correlated directly with the median BNF rates measured. These $\delta^{15}N$ values showed a significant negative correlation with the atmospheric Nr deposition where the values increased (from -5.73 ‰ in Fenn's & Whixall to -1.49 ‰ in Forsinard) as the Nr deposition decreased (from 27 in Fenn's & Whixall to 6 kg N ha^{-1} yr^{-1} in Forsinard), which is in line with the findings of Zivkovic et al. (2017) in Canada. Additionally, it was discovered a significant negative correlation between the NH_4^+:NO_3^- ratio and the $\delta^{15}N$ signature at all the sites which is in agreement with the findings of

Bragazza et al. (2005). These results suggest that the higher Nr deposition rates imply a higher availability of NH_4^+-N that is initially filtered by the mosses and this source of N leads toward more negative $\delta^{15}N$ values, playing BNF a less important role as N source. As the ratio (NH_4^+:NO_3^-) decreases from the south to the north (F&Whixall >Migneint>Forsinard>Degerö), and with it the amount of Nr deposition, the NO_3-N presence increases which leads to more positive values of $\delta^{15}N$, and at the same time BNF plays a more significant role as N source, as corroborates the significant correlation, and being the $\delta^{15}N$ values closer to 0‰ (Stewart et al. 2011b). In Degerö, with almost no anthropogenic atmospheric Nr deposition, the lower $\delta^{15}N$ values than in Forsinard, could be due to a major role of the N uptake by mosses from peat decomposition that leads to $\delta^{15}N$ depletion (Knorr et al., 2015; Zivkovic et al., 2017). It was also detected that regarding species *Sphagnum* mosses had lower $\delta^{15}N$ values than peat, and that within the *Sphagnum* mosses species forming hummocks had lower $\delta^{15}N$ values than species in hollows, which could be explained as a result of different ways of obtaining the N by the species: the ones in hummocks absorb the N mainly from Nr deposition that contains more depleted $\delta^{15}N$ values, while the species in hollows may combine the uptake with N from the soil and the groundwater as well that have less negative $\delta^{15}N$ values (Asada et al., 2005); and the peat $\delta^{15}N$ values closer to 0‰ suggest that the N comes from a combination of ways playing BNF an important role (Knorr et al., 2015).

The results show that high rates of chronic Nr deposition, and also decades of high rates of S and T treatments, does not shut down BNF in *Sphagnum*-dominated peatlands completely. Other studies have reported that high rates of Nr deposition reduced the *Sphagnum* cover up to 84% (Berendse et al., 2001; Wiedermann et al., 2007; Erikson et al., 2010a; Levy et al., 2019), because of changes in the peat biogeochemistry (Bragazza and Limpens, 2004; van den Elzen et al., 2018) and thus an increase in vascular plants (Lamers et al., 2000; Bragazza

et al., 2012). It was also found that while the percentage of reduction in BNF with a high dose of S (20 kg ha^{-1} yr^{-1}) was only of a 33% and with a high dose of N (30 kg ha^{-1} yr^{-1}) of a 64%, the combination of low doses of N and S (15 and 10 kg ha^{-1} yr^{-1} respectively) did reduce BNF by 87%, high doses of N and S (30 and 20 kg ha^{-1} yr^{-1} respectively) by 89%, and high doses of N and S plus T by 94% (Fig. 3.9). These findings can explain why Zechmeister-Boltenstern and Kinzel (1990) have found in Central Europe BNF rates of about 12.17 nmol N gDW^{-1} d^{-1} which are very low. The reason could be that Central Europe, particularly the industrialised areas close to the main cities, suffered from heavy pollution, not only with high atmospheric Nr deposition (at the north-east of Austria, for 1991, ~21 kg N ha^{-1} yr^{-1}) but also with high atmospheric S deposition (19.1 kg S ha^{-1} yr^{-1}; Puxbaum and Gregori, 1998), and while the addition of sulphur alone may promote BNF due to an increase in sulphate-reducing bacteria, that has been reported to contribute between 20 and 53 % to BNF rates in some wetlands (Santruckova et al., 2010), it is the combination of N, S, and T that may reduce BNF up to ~94%.

These results showed that BNF in peatlands with high rates of atmospheric Nr deposition (17-27 kg N ha^{-1} yr^{-1}) contributed to their ecosystems with up to 5.5 kg N ha^{-1} per growing season which is within the range of previous studies (1-10 kg N ha^{-1} yr^{-1}) conducted in peatlands subject to high rates of Nr deposition (>10 kg N ha^{-1} yr^{-1}) in the USA (Chapman and Hemond, 1982; Urban and Eisenreich, 1988). Moreover, the contribution of BNF in pristine peatlands increased significantly to 16.3 kg N ha^{-1} per growing season, which is in agreement with the results found in other studies in pristine locations in Finland (1-29 kg N ha^{-1} yr^{-1}; Larmola et al., 2014) and in Canada (4.8-62.3 kg N ha^{-1} yr^{-1}; Vile et al., 2014).

It can be concluded that BNF did not shut down under chronic high rates of Nr deposition. In fact, these results showed that the suppression effects of the Nr deposition (rate of BNF reduced per unit of Nr deposition) decreased as the Nr deposition rates increased following

a power equation ($R^2 = 0.99$). Similarly, in the British sites, the $\delta^{15}N$ signature increased as the Nr deposition decreased, indicating a higher role of BNF as source of N. Also, it was found differences *on site*, where species in hollows fixed, on average, 61% more than species in hummocks, because the Nr deposition can have a more direct effect on the latter and the wetter conditions of the former. BNF contribution needs to be accounted for, not only in pristine peatlands, but also in the polluted areas, when modelling the N economy of peatlands. More work is needed to understand the biotic and abiotic controls of BNF at the landscape scale to be able to effectively include BNF in N models.

3.6 References

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3.7 Supplementary material

Table 3.S1. Elements in *Sphagnum* mosses. Data shown is median (±MAD) per site (n=12). Sites with different letters are significantly different.

	Fenn's&Whixall	Migneint	Forsinard	Degerö
Elements	µg/g (±MAD)	µg/g (±MAD)	µg/g (±MAD)	µg/g (±MAD)
Mg	**512.86** (±136.23) a	**1111.68** (±143.27) b	**1238.57** (±101.75) b	**383.20** (±103.09) a
K	**4283.18** (±1243.21) a	**2497.15** (±920.92) b	**2344.12** (±771.34) b	**3071.33** (±522.60) ab
Ca	**305.29** (±90.02) a	**428.71** (±387.29) a	**428.09** (±132.73) a	**251.39** (±161.13) a
V	**79.37** (±0.79) a	**78.41** (±1.26) b	**73.90** (±4.17) b	**74.27** (±0.48) b
Mn	**83.25** (±18.08) a	**79.80** (±30.94) a	**31.83** (±4.32) a	**41.47** (±41.47) a
Co	**19.42** (±0.31) a	**19.61** (±0.70) ab	**16.46** (±1.83) bc	**13.17** (±0.11) c
Ni	**296.10** (±1.67) a	**293.27** (±4.15) b	**246.73** (±1.46) bc	**244.84** (±1.21) c
Cu	**55.88** (±0.52) a	**55.68** (±0.97) a	**48.08** (±0.98) a	**<LOD**
Mo	**331.39** (±1.96) a	**331.34** (±8.08) ab	**287.07** (±4.33) b	**286.34** (±1.49) b
	mg/g (±MAD)	mg/g (±MAD)	mg/g (±MAD)	mg/g (±MAD)
C	**441.32** (±4.16) a	**435.90** (±4.46) b	**442.13** (±5.62) ab	**441.03** (±2.52) a
N	**5.57** (±0.68) a	**5.48** (±0.55) a	**6.88** (±1.64) a	**5.20** (±0.60) a
P	**0.41** (±0.03) a	**0.34** (±0.05) b	**0.34** (±0.12) b	**0.29** (±0.02) c
Ratios				
C:N	**79.68** (±11.46) a	**79.91** (±9.06) a	**65.24** (±14.05) a	**85.79** (±9.63) a
C:P	**1046.29** (±74.30) a	**1333.27** (±188.40) bc	**1265.01** (±646.57) b	**1537.57** (±85.49) c
N:P	**11.99** (±0.73) a	**16.39** (±1.43) a	**20.75** (±3.83) a	**17.00** (±1.69) a

Table 3.S2. Elements in peat. Data shown is median (±MAD) per site (n=6). Sites with different letters are significantly different.

	Fenn's&Whixall	Migneint	Forsinard	Degero
Elements	µg/g (±MAD)	µg/g (±MAD)	µg/g (±MAD)	µg/g (±MAD)
Mg	**480.21** (±33.05) a	**335.31** (±26.44) b	**1044.35** (±35.43) a	**314.41** (±85.16) b
K	**<LOD**	**33.91** (±12.45) a	**61.14** (±9.67) ab	**165.98** (±21.48) b
Ca	**922.40** (±42.29) a	**139.53** (±63.14) b	**197.76** (±57.11) bc	**413.19** (±149.03) ac
V	**114.90** (±1.88) a	**79.58** (±1.01) b	**69.63** (±0.94) b	**74.59** (±0.81) b
Mn	**<LOD**	**<LOD**	**<LOD**	**<LOD**
Co	**27.53** (±0.80) a	**21.75** (±0.39) a	**17.84** (±0.14) ab	**13.05** (±0.17) b
Ni	**417.10** (±5.99) a	**291.85** (±4.95) ab	**244.18** (±0.10) ab	**239.46** (±5.51) b
Cu	**289.80** (±75.85) a	**172.95** (±28.40) b	**113.25** (±29.20) b	**16.69** (±10.94) b
Mo	**465.02** (±6.79) a	**326.94** (±4.02) ab	**283.11** (±8.33) ab	**281.37** (±4.17) b
	mg/g (±MAD)	mg/g (±MAD)	mg/g (±MAD)	mg/g (±MAD)
C	**512.26** (±1.38) ac	**465.36** (±0.77) b	**533.33** (±1.23) c	**481.21** (±1.82) ab
N	**12.23** (±0.02) ab	**17.00** (±0.07) c	**15.79** (±0.02) bc	**10.76** (±1.77) a
P	**0.29** (±0.01) a	**0.77** (±0.02) b	**0.29** (±0.00) a	**0.54** (±0.13) ab
Ratios				
C:N	**41.80** (±0.03) a	**27.37** (±0.16) b	**33.83** (±0.14) ab	**45.44** (±7.62) a
C:P	**1745.04** (±18.31) ab	**607.77** (±9.18) c	**1852.65** (±16.86) a	**951.04** (±240.10) bc
N:P	**40.30** (±0.33) ab	**22.20** (±0.46) b	**54.93** (±0.07) a	**21.96** (±8.72) b

CHAPTER 4: RESPONSE OF BIOLOGICAL NITROGEN FIXATION IN *SPHAGNUM* MOSSES AND PEAT EXPOSED TO EXCESSIVE NUTRIENTS AND MICROBIAL RESPIRATORY METABOLITES

4.1 Abstract

Aims: During the last decades, temperate peatlands have been subject to chronic and high rates of atmospheric reactive nitrogen (Nr) deposition along with other pollutants. At the same time, there was a massive increase in CO_2 concentration in the atmosphere. There have been studies looking at the effects of N, P, K, and Mg on plant performance and of elevated CO_2 on photosynthesis, but not on their effects together on BNF.

Methods: The BNF rates were determined using the $^{15}N_2$ direct assimilation method. *Sphagnum* mosses were collected from hollows (*S. cuspidatum*) and from hummocks *(S. papillosum*) as well as bulk peat (0-15 cm) in a peatland with low atmospheric Nr deposition. Two mesocosm experiments were created. In one it was applied available N, a total amount of 1091 µg N per pot, in doses of 136 µg N every two days for a 16 days period (6 of acclimatisation + 10 of treatment) as background, to saturate the mosses with N. Following,

four different nutrient treatments were applied: 60.5 µg P, 605.2 µg P, 121 µg K, and combined 605.2 kg P and 121 µg K per pot, five times in doses of one fifth each. In the second, it was applied a background of 1091 µg N plus combined 605.2 µg P and 121 µg K per pot as indicated before. Following, three different treatments 121 µg Mg per pot, elevated microbial respiratory metabolites (MRMs) (CO_2, N_2O and CH_4), and combined 121 µg Mg per pot and elevated MRMs, as in the first experiment.

Results: High rates of Nr addition reduced BNF rates by 83% and low addition of P stimulated BNF rates by 59%, high P by 34%, and the combination of P and K increased BNF by 24%. The addition of Mg under N, P, and K saturation resulted in an increase of BNF rates by 198% and elevated MRMs by 533%, but their combination only by 128%. BNF rates were highly variable and significantly different among species.

Conclusions: BNF activity does not shut down under high rates of Nr saturation. When N is not a limitation for BNF activity, it is co-limited by P&K. Under no limitation of N, P, and K to boost BNF other elements come into play such as Mg that is essential in the BNF process, and elevated MRMs as CH_4 and N_2O that provide energy, and CO_2 that feeds photosynthesis.

4.2 Introduction

Biological nitrogen fixation (BNF) plays a key role in peatlands ecology, particularly in ombrotrophic bogs that under pristine conditions are nutrient-poor ecosystems relying on atmospheric deposition for their nutrient acquisition (Aerts et al., 1992; Vile et al., 2014; Knorr et al., 2015). Peatlands have paramount importance globally as they are carbon (C) and nitrogen (N) sinks. In fact, they cover just 3% of the Earth's terrestrial surface (Clymo, 1984b), but store approximately 33% of the global soil C (Knorr et al., 2015) and up to 16% of the global soil N (Vile et al., 2014). This C and N storage is possible due to a variety of factors that define most peatlands such as nutrient-poor (Berg et al., 2013), acidic (Clymo, 1984a), and waterlogged areas that prevent organic matter from total decomposition and thus it accumulates organic C in the form of peat (Lindsay, 1995). However, during the past century there has been an overwhelming introduction of anthropogenic reactive N (Nr) into the biosphere due to the Haber-Bosch synthetic fertilizer production and to a substantial increase of fossil fuel combustion which releases nitrogen oxides into the air (Vitousek et al., 2013). Ammonia volatilization from agriculture and nitrogen oxides emission from fossil fuel combustion are deposited back including in peatlands and other natural terrestrial ecosystems. Nr deposition in the UK substantially increased since the mid of the twentieth century and it peaked around 1990 with an average maximum for the whole country rate of >30 kg N ha^{-1} yr^{-1} that has been falling ever since, and that will continue falling further by 2030, in general, but stabilising at values close to the ones of the 1960s (Payne, 2014).

High atmospheric Nr deposition can have negative consequences for peatlands, and in some European countries, at the beginning of this century, it has reached rates of >80 kg N ha^{-1} yr^{-1} (Novak et al., 2015). Bragazza et al. (2004) reported that Nr deposition rates over 10 kg ha^{-1} yr^{-1} can shift *Sphagnum* mosses from being limited by N to being co-limited by P and K. The moss carpet gets N saturated and stops its filter function allowing N to leak into the peat

layer, and thus, boosting vascular plants (Lamers et al., 2000). It can change its biogeochemistry and increase the decomposition rates shifting from being C and N sinks to sources (Gunnarsson and Rydin, 2000); it also downregulates BNF and can even inhibit it (Rousk et al., 2014). Phosphorus thus becomes a key element when N is no longer scarce, particularly for N_2-fixers that are more P demanding, in part because BNF is an energy-intensive process that needs a large amount of ATPs to support N_2 reduction to NH_3 (Vitousek et al., 2002). In boreal ecosystems (low atmospheric Nr deposition rates) BNF is not affected by P (Rousk et al., 2017). On the other hand, in temperate peatlands, van den Elzen et al. (2017) found that under high rates of Nr deposition (~25 kg ha^{-1} yr^{-1}) the addition of P resulted in doubling BNF rates, however, they found no effect on moss growth, and they did not test the possibility of being K limited as well.

The presence of K is essential for plant growth, and it plays a key role in two processes that affect BNF, which includes nutrient transport in cells and, linked to photosynthesis, the production of ATP (Sarkkola et al., 2016). K is highly soluble in water, and even more in acidic conditions, so in peatlands, that are mostly acidic, it is easy to be leached out. Even though P and K can be found in peat, it does not mean that these nutrients are available for plant uptake due to its adsorption to organic matter (Øien and Moen, 2001). In fact, a study in a forested peatland in Canada reported K deficiencies for vascular plants (Bhatti et al., 1998). Similarly, high rates of Nr deposition are linked to a lower retention of Ca and Mg as a way to alleviate intoxication through these highly exchangeable ions by bryophytes (Soares and Pearson, 1997). And Mg is an essential element not only for BNF (it joints ATP or ADP to intervene in the reduction process of N_2), but also for photosynthesis (it promotes different reactions; Lea and Leegood, 1993).

Peatlands, which are predominantly water-saturated produce a substantial amount of CH_4 due to methanogenesis and CO_2 during microbial respiration. Being wet, the porewater of

peatlands often reach a high concentration of these gases compared to atmospheric concentrations. Temperate peatlands which are exposed to high Nr deposition also produces N_2O due mainly through denitrification (Sgouridis and Ullah, 2017; Pärn et al., 2018). Such high concentration of these greenhouse gasses in the pore water and air of peatlands can potentially affect BNF through, a) provision of CH_4 for methanotrophic oxidation to generate energy for fuelling BNF, and b) and enhancement of photosynthesis by mosses under elevated CO_2 concentration (Porada et al., 2013). Therefore, it is likely that high CH_4 and CO_2 concentrations can significantly enhance BNF to meet microbial and plant N demands. Recently, the respiration reduction of N_2O to N_2 by nitrous oxide reduction enzymes has been reported to affect BNF through a) provision of energy by reduction of N_2O to N_2 by microbes, and b) by providing N_2 to diazotrophs under saturated conditions for its fixation back to NH_3 (Farias et al., 2013); although there are no conclusive studies about the former. A study also reported that N_2O reduction is directly linked to BNF suggesting that N_2O is reduced for direct assimilation in BNF (Desloover et al., 2014). Therefore, the presence of an elevated concentration of CH_4, N_2O and CO_2 could affect BNF. It also would be affected by the fact that high rates of Nr deposition would cause shifts in peatlands vegetation composition from a dominance of *Sphagnum* mosses to vascular plants because of changes in the biogeochemistry of the area, and one of the changes would be lower rates of C sequestration (Berendse et al., 2001). However, a study done on *Sphagnum* mosses found that elevated CO_2 stimulated photosynthesis, but just for three days, suggesting that the boost is not permanent (Van Der Heijden et al., 2000), or that it is not significant to induce a difference in BNF activity in mosses of arctic and subarctic ecosystems (Rousk et al., 2016). In laboratory incubations of bacterial cultures from forested peatlands, BNF rates were higher with elevated CO_2 (Lindo and Griffith, 2017), but in collected peat samples BNF rates were unaffected by CO_2 as well as by CH_4 (Warren et al., 2017). So, the effects of increased

microbial respiratory metabolites (MRMs) on BNF are not clear. In addition, it is critical, as it is a possible future scenario that has not been studied before, to evaluate BNF activity under Nr saturation conditions and under an increase of greenhouse gasses that are likely in the future (Pärn et al., 2018).

This study had two key objectives that were investigated through two fertilization experiments in the laboratory, with *Sphagnum* mosses and peat collected from a peatland with relatively low Nr deposition rates. The first objective was to evaluate the short-term effects of Nr saturation of peatlands on BNF, followed by the addition of P, and K. The second was to evaluate BNF under the previous conditions (Nr, P, and K) and the short-term response to the addition of Mg and elevated MRMs (CO_2, CH_4, N_2O). It was hypothesised that under chronic increased Nr deposition BNF activity would decrease; but that under these Nr saturated conditions the addition of P and K would increase BNF activity, and that under the saturation of Nr, P, and K the addition of Mg and MRMs would further increase BNF activity.

4.3 Material and methods

4.3.1 Sample collection

Two different *Sphagnum* moss species, one located in hollows *S. cuspidatum*, and one located in hummocks, *S. papillosum*, and bulk peat (0-15 cm) were collected from a peatland in Forsinard (Scotland) in June 2017. Peat collected at the mosses same spot was used to grow them again on it in the laboratory during the experiments. Samples of the mosses and peat were collected by hand and placed in a cool box for transportation to the laboratory at Keele University (England). The location had a mean annual temperature of 6.9 ºC, a mean annual precipitation of 1104 mm, and an atmospheric Nr deposition of < 6 kg ha^{-1} yr^{-1} (Saiz

et al., 2019). This site was selected because it has one of the lowest Nr deposition rates in the UK and because of the low anthropogenic disturbances (it is in a National Nature Reserve). It was measured BNF *in situ* to have a reference of BNF rates under field natural conditions.

4.3.2 Experimental design

Immediately after the collection of the mosses and peat from the field the samples were refrigerated (~4 ºC) for a few weeks until the experiments started in July. The collected mosses were washed with deionized water and placed in pots that were 8.5 cm height and a diameter of 7.5 cm (a surface of 44.18 cm^2), and that contained a bottom layer (20 mm) of their own peat. For the peat samples, the peat layer was double (40 mm) to have enough sample for incubations, and the pots were covered with aluminium foil to avoid light through the pot. Then *Sphagnum* moss was also placed in the pots to mimic natural conditions (Fig. 4.1).

Prior to the treatment additions, the samples were acclimatized to the laboratory conditions for 7 days. The temperature was about 20 ºC ±2; the light cycle was 15h day and 9h night and during the day the photosynthetically active radiation range was between 265 and 771 µmol m^{-2} s^{-1}. Mosses were watered according to the precipitation of the area every two days. At the same time the background treatments started for each of the two sets of experiments. For the P & K treatments experiment, the background additions consisted of a total of 1091 µg N per pot in the form of NH_4NO_3 to ensure that mosses were saturated with N. It was applied in doses of 136.38 µg N per pot a total of 8 times, three during this acclimatization period and five during the following treatments period. It was dissolved in water and applied through the watering. And for the Mg and MRMs treatment additions the background

consisted of 1091 μg N plus 605.2 μg of P, in the form of NaH_2PO_4 and 121 μg of K, in the form of KNO_3, total per pot which was applied dissolved in water as the previous one, i.e. in doses of 1/8 a total of 8 times (Fig. 4.2).

The P & K experiment consisted of 5 treatments (Fig. 4.2): control (background mentioned above); 60.5 μg P per pot; 605.2 μg P per pot; 121 μg K per pot; and 605.2 μg P and 121 μg K per pot. Each treatment was done on 3 replicates for the two different moss species and peat. They were added in the water, simulating rain, in the amount of one fifth every two days so the addition lasted 10 days (A total of five doses). The total number of pots was 60 (3 replicates + 1 control x 5 treatments x 3 species).

The Mg and MRMs experiment consisted of 4 treatments (Fig. 4.2): control (background mentioned above); 121 μg Mg per pot in the form of $MgSO_4$; incubation under elevated MRMs: 520 ppm CO_2, 1.26 ppm N_2O, and 3.44 ppm CH_4; and 121 μg Mg per pot and incubation under elevated MRMs. The MRMs treatment was applied as 10% of the total volume of the vial which was 50 ml. So 5 ml of the gas mixture was injected at the same time that the $^{15}N_2$ gas incubations with the final concentrations (ppm) in the vial mentioned above. The gas treatment was simultaneous to the BNF incubations. Ambient air concentrations of the MRMs were: 400 ppm CO_2, 0.33 ppm N_2O, and 1.8 ppm CH_4. The treatments (no gaseous) were applied on 3 replicates in the form of simulated rain every two days, so one fifth each time until it was reached the total amount equivalent for pot. The total number of pots was 48 (3 replicates + 1 control x 4 treatments x 3 species).

Figure 4.1. Photographs of the two sets of experiments: upper photograph of the P & K fertilization experiment; and bottom photograph of the Mg and MRMs fertilization experiment.

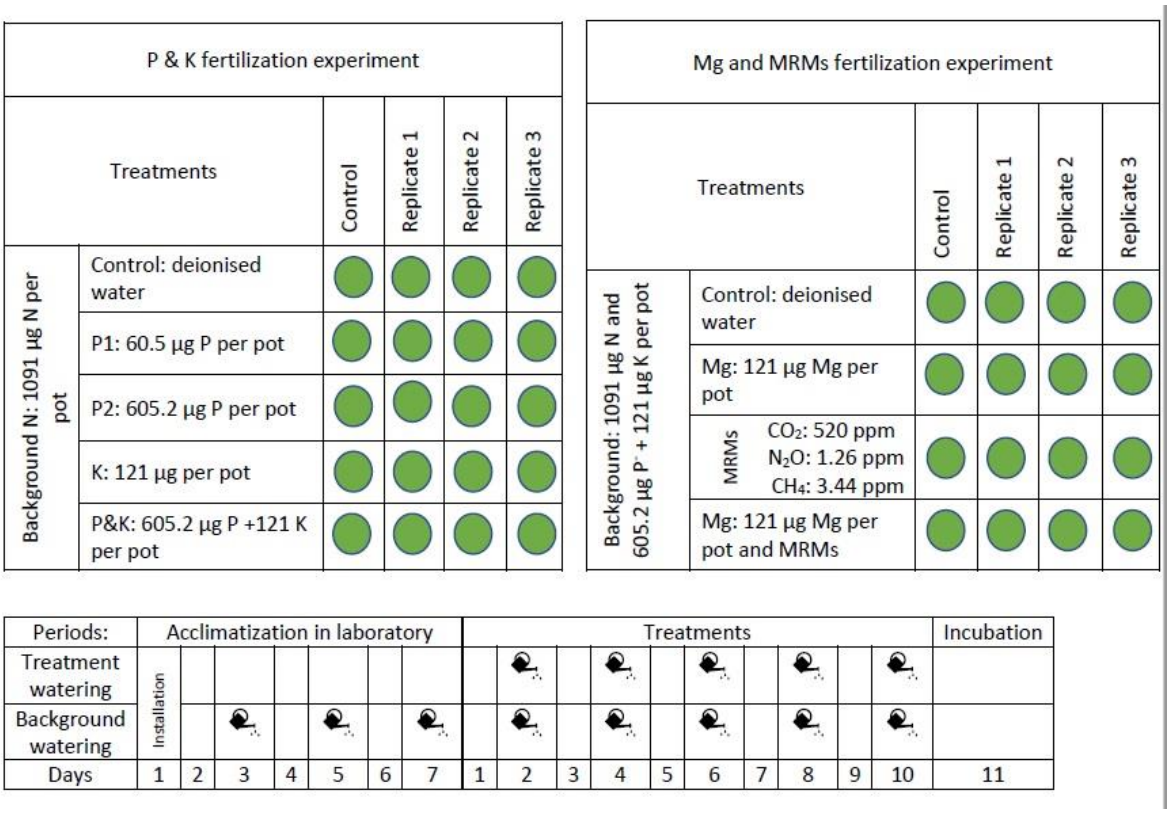

Figure 4.2. Sketch of the two sets of experiments and timeline of the background and treatment watering.

4.3.3 $^{15}N_2$ direct assimilation method

BNF was measured using the $^{15}N_2$ direct assimilation method as in Saiz et al. (2019). For each of the *Sphagnum* species and peat and for each treatment three out of the four replicates were incubated with $^{15}N_2$ (98 atom% Cambridge Isotope Laboratories Inc., USA), with the fourth being the control (only air added). From each pot, about 20 shoots of *Sphagnum* moss (upper 5 cm) or 10 g of peat (after passing it through a 2 mm sieve) were put into 50 ml vials (Fig. 4.3). They were closed with gas-tight rubber septa and located in the same spot the pots were to maintain similar conditions of light and temperature mentioned before, except for peat samples that were placed in a dark box to avoid light. After closing the vials, 5 ml of air were evacuated (10 % of the headspace), replaced by $^{15}N_2$ (98 atom%) gas and incubated

for 24 hours. In the case of the MRMs treatment, it was evacuated 10 ml of air to replace it by 5 ml of MRMs first, and then the 5ml of $^{15}N_2$ gas.

Figure 4.3. Bottles prepared with *Sphagnum* mosses from the Mg and MRMs fertilization experiment.

Immediately after the 24-hour incubation the vials were opened and aerated to allow $^{15}N_2$ remaining gas to escape. Just next, the opened vials were weighed and placed into the oven for drying at 70 ºC for 72 hours. Once dried, the samples were weighed again to calculate gravimetric moisture. Then, they were pulverised, and subsamples were sent to the Life Sciences Mass Spectrometry Facility, Centre for Ecology and Hydrology, at Lancaster for total N and ^{15}N content analysis by Isotope Ratio Mass Spectrometry (IRMS) using an elemental analyser (Carlo Erba NA1500, Italy) coupled to an isotope ratio mass spectrometer (Dennis Leigh Technologies, UK). The analytical precision was 0.36 ‰, and the contamination of the $^{15}N_2$ gas (Dabundo et al., 2014) was corrected using -0.03 ‰ as a threshold (calculated by testing the gas) below which any difference between the control and

enriched samples were not considered significant (see Saiz et al., 2019 for more details). The BNF rates were calculated using the formula (Equation 4-1; Liengen, 1999):

$$Y = \left(\frac{atom\%\ ^{15}Nexcess}{100}\right) x \left(\frac{totalNsample\ x\ 10^{9}}{t\ x\ 28}\right) x \left(\frac{100}{\%^{15}N\ headspace}\right)$$

where Y (nmol N gDW^{-1} h^{-1}) is the amount of N_2 fixed during the experiment, atom% $^{15}Nexcess$ is the difference between *atom%*^{15}*Nsample* and *atom%15Ncontrol*, total N is the total amount of nitrogen in the sample (g N 100 gDW^{-1}), t is the incubation time, 28 is the molecular weight of N_2 (g mol^{-1}), and *%*^{15}*N headspace* is the percentage of ^{15}N out of the total amount of N gas in each incubation bottle.

4.3.4 Statistical analysis

The data obtained was tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's Test). Non-parametric tests were used to analyse the data. To test the effects of the different treatments on different variables (e.g. BNF, total N) the Kruskal-Wallis test followed by pairwise comparisons was used. To test the correlation between two variables (e.g. BNF, total N) the Spearman's rho correlation coefficient was used. Significant differences were considered when $P < 0.05$. All data was analysed using IBM statistic software SPSS version 24.

4.4 Results

In the laboratory, the addition of 1091 µg N per pot resulted in an overall suppression of the BNF rates by 86.36% considering the median, which was significant ($P < 0.05$). This result was obtained by comparing the BNF results in the laboratory with the ones measured in the field, in Forsinard, under an Nr deposition rate < 6 kg N ha^{-1} yr^{-1}. Under this background of

1091 µg N per pot, the addition of 60.5 µg P per pot resulted in a median increase of the BNF rates by 59%, while the addition of 605.2 µg P per pot just by 34% (taking the mean value it was by 249%), and the addition of 121 µg K per pot by 12%. The addition of a combination of 605.2 µg P per pot and 121 µg K per pot resulted in a significant increase of the BNF rates by 487% ($P < 0.05$; Fig. 4.4). The results of the BNF rates by species in this P & K treatment experiment showed that *S. papillosum* had the lowest median fixation value, and peat had the highest one, however, the maximum single value of the rates was obtained by *S. cuspidatum* suggesting the possibility of a hot spot in one of the replicates (Fig. 4.5).

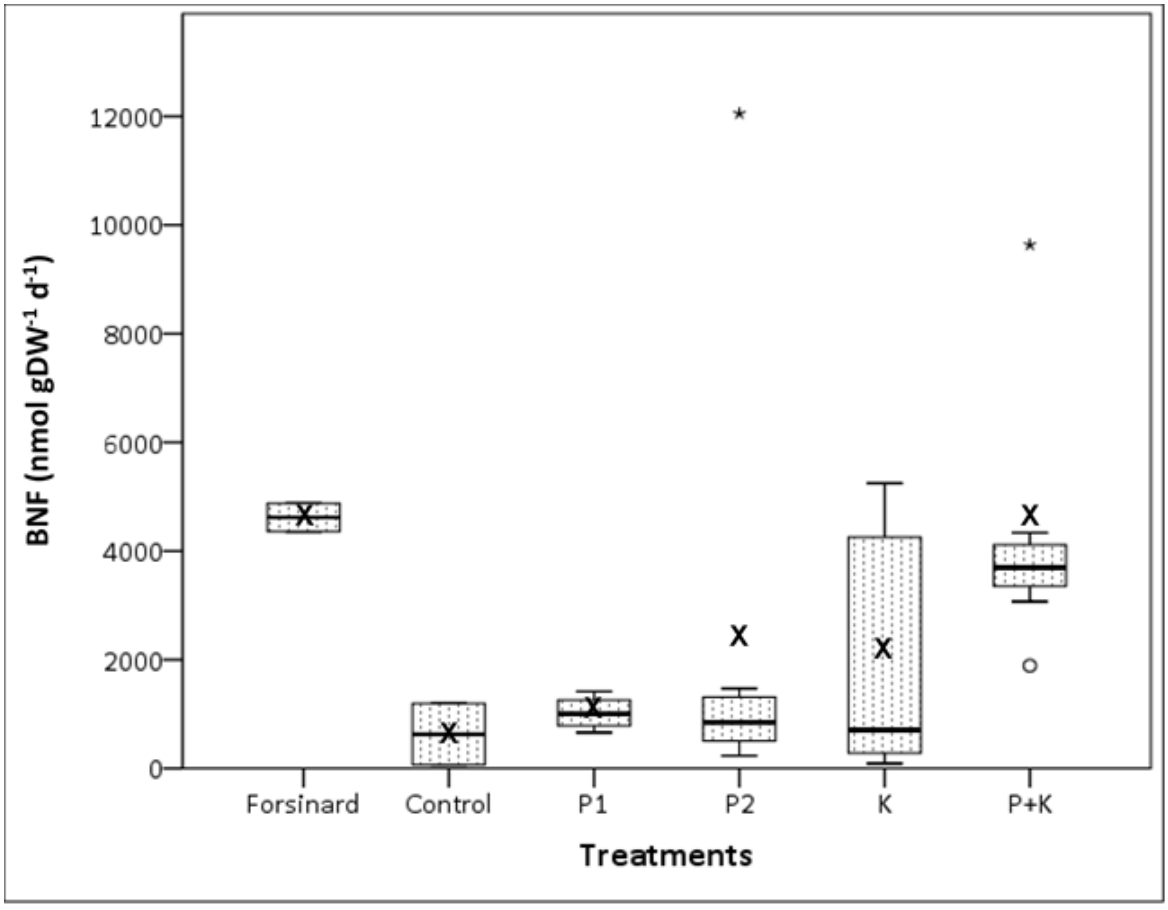

Figure 4.4. BNF rates (nmol gDW^{-1} d^{-1}) by treatment. Forsinard: field incubations under atmospheric Nr deposition rates of < 6 kg ha^{-1} yr^{-1}. Control: Background of 1091 µg N per pot for N saturation. P1: background plus 60.5 µg P per pot. P2: background plus 605.2 µg P per pot. K: background plus 121 µg K per pot. P+K: background plus 605.2 µg P and 121 µg K per pot. The box indicates the 25th percentile, the median (central line), and the 75th percentile. The whiskers show the maximum and minimum values. The X sign indicates the mean value. The dot is an outlier (< 1.5 IQR), and the stars are extreme values (> 3 IQR). (n = 3)

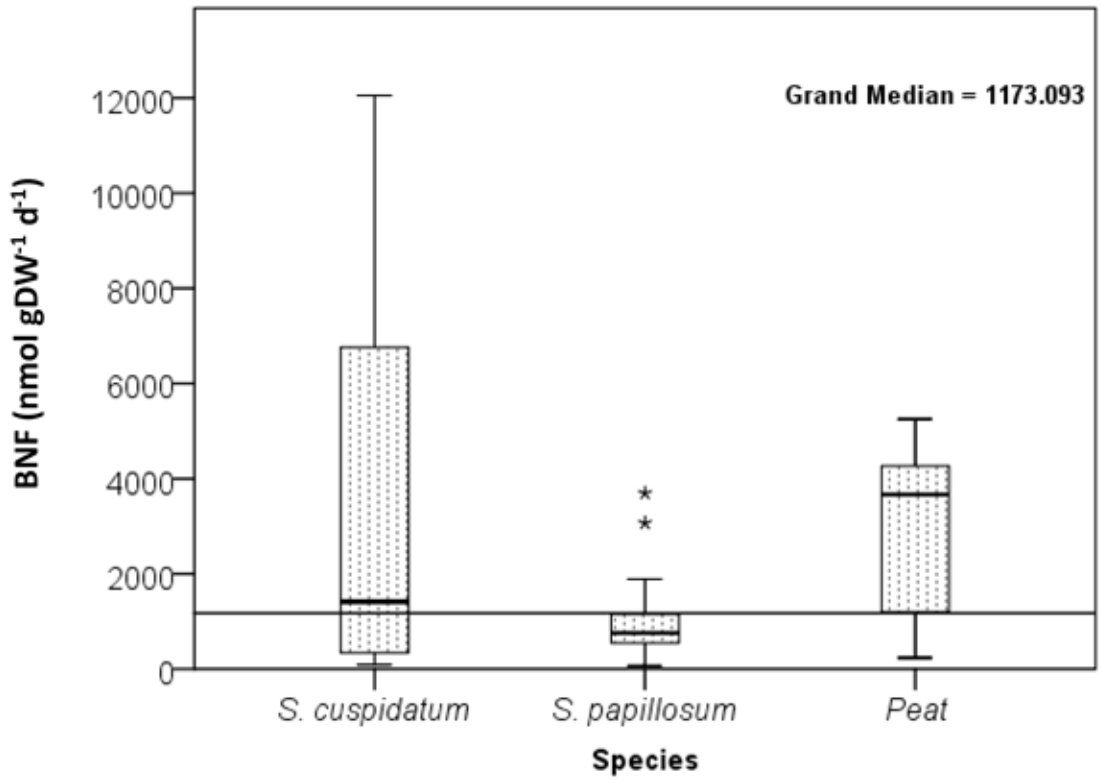

Figure 4.5. BNF rates (nmol gDW^{-1} d^{-1}) by species under the different P & K treatments. The box indicates the 25th percentile, the median (central line), and the 75th percentile. The whiskers show the maximum and minimum values. The stars are extreme values (> 3 IQR). (n = 15)

In a parallel experiment in the laboratory, the addition of 1091 μg N per pot plus 605.2 and 121 μg per pot of P and of K respectively resulted in an overall suppression of the median BNF rates by 83% (while taking the mean values it was by 50%, thus being closer to the P&K experiment values). With this background the addition of 121 μg Mg per pot resulted in an increase of the median BNF rates by 198%, the addition of MRMs in an increase by 533%, but the addition of 121 μg Mg per pot and MRMs only by 128% although, considering mean rates, the increase was by 3025% (Fig. 4.6). In this experiment, the BNF results by

species (Fig. 4.7) showed that *S. cuspidatum* got the highest median BNF rates as well as the maximum value that were triggered mainly by the addition of MRMs, indicating again the existence of a hot spot.

Moisture did not affect significantly BNF rates in any treatment ($P > 0.05$), although significant differences were found between *Sphagnum* species from hollows (wetter) and hummocks (less moisture), and peat ($P < 0.001$). *Sphagnum* species from hollows had the highest median moisture content (%), while peat had the lowest (Fig. 4.8).

The different treatments did not affect significantly the total N content ($P > 0.05$) in *Sphagnum* species and peat. However, the results showed a significant positive correlation between BNF rates and total N content (%) in both set of experiments ($P < 0.05$): P & K treatments and Mg & MRMs treatments. The results also showed a significant difference ($P < 0.01$) in the total N content between *Sphagnum* species under both treatments (Fig. 4.9a and Fig. 4.9b), presenting the hollows one (*S. cuspidatum*) higher N content, about 20% more in both cases, than the hummocks one (*S. papillosum*).

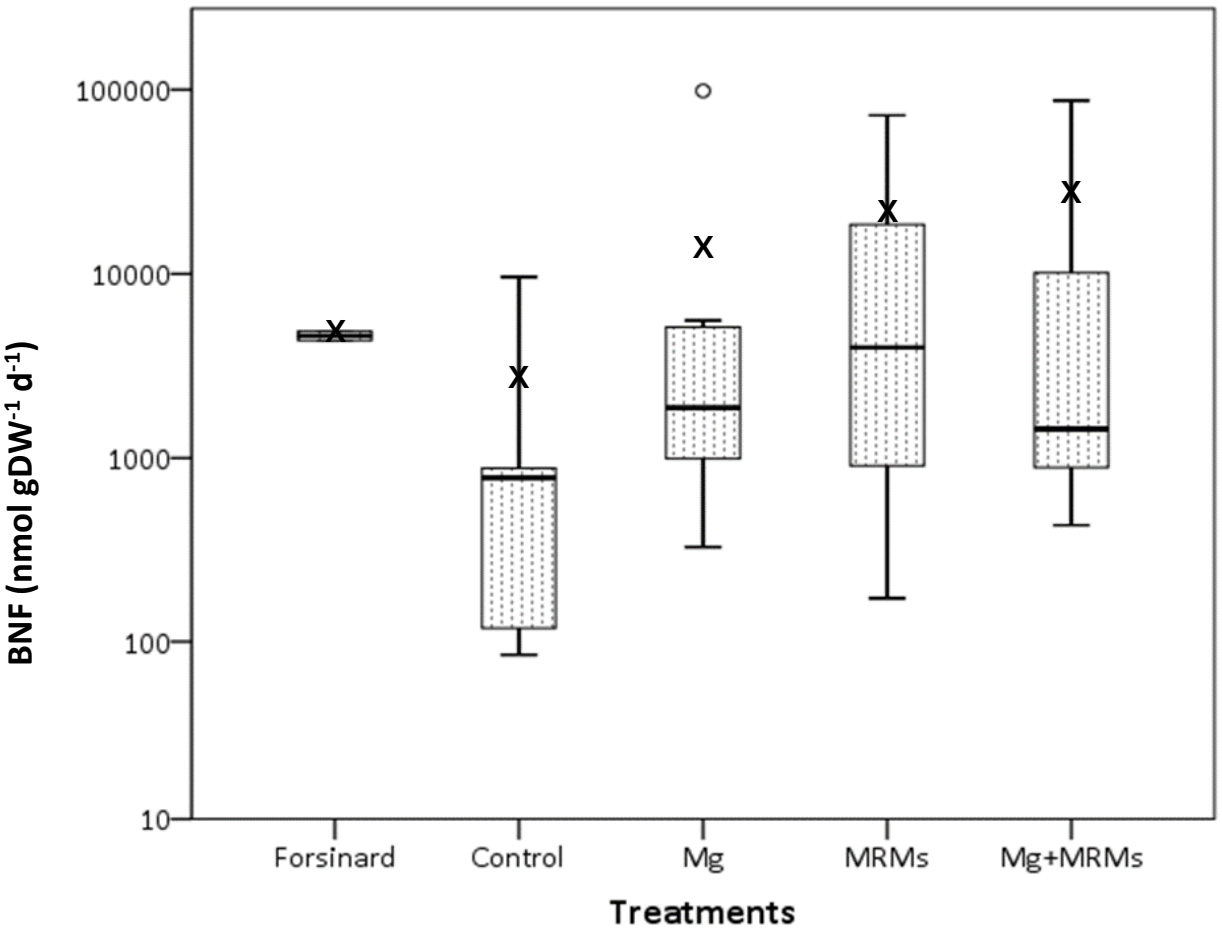

Figure 4.6. BNF (nmol gDW^{-1} d^{-1}) by treatments of Mg & MRMs. Forsinard: field incubations under atmospheric Nr deposition rates of < 6 kg ha^{-1} yr^{-1}. Control: Background of 1091 µg N per pot plus P and K 605.2 and 121 µg per pot respectively. Mg: background plus 121 µg Mg per pot. MRMs: background plus MRMs. Mg+MRMs: background plus 121 µg Mg per pot and MRMs. The box indicates the 25th percentile, the median (central line), and the 75th percentile. The whiskers show the maximum and minimum values. The X sign indicates the mean value. The dot is an outlier (> 1.5 IQR). Note the log-scale y-axis (n = 3).

The $\delta^{15}N$ signature was not affected by any of the different treatments. The $\delta^{15}N$ signature of the peat was positive, with a median of 1.72‰ for the P & K treatments and 2.67‰ for the Mg & MRMs treatments. On the other side, the negative one, the $\delta^{15}N$ signature of the *Sphagnum* species were: -1.43‰ and -1.55‰ for *S. papillosum* and -1.43‰ and -1.15‰ for *S. cuspidatum* regarding the P & K and Mg & MRMs treatments respectively (Fig. 4.10). *Sphagnum* species $\delta^{15}N$ signature was closer to zero, indicating that BNF was the main source of N.

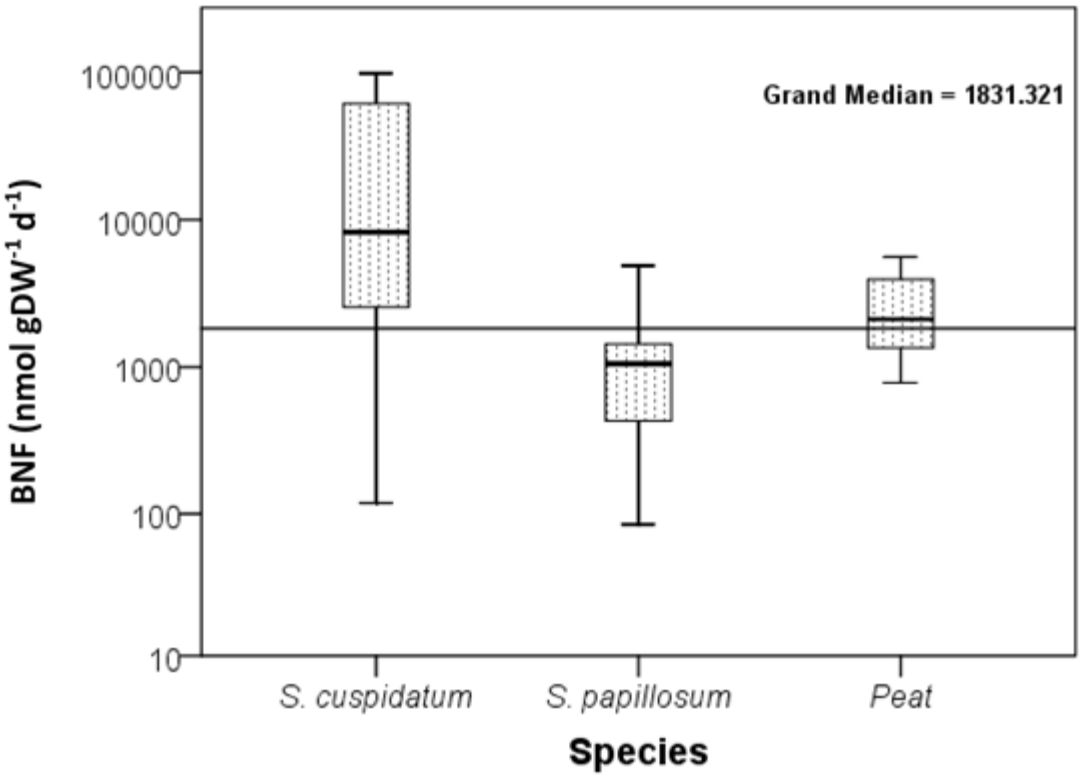

Figure 4.7. BNF (nmol gDW^{-1} d^{-1}) by species under the Mg & MRMs treatments. The box indicates the 25th percentile, the median (central line), and the 75th percentile. The line across the graph indicates the median of all the data. The whiskers show the maximum and minimum values. Note the log-scale y-axis (n = 12).

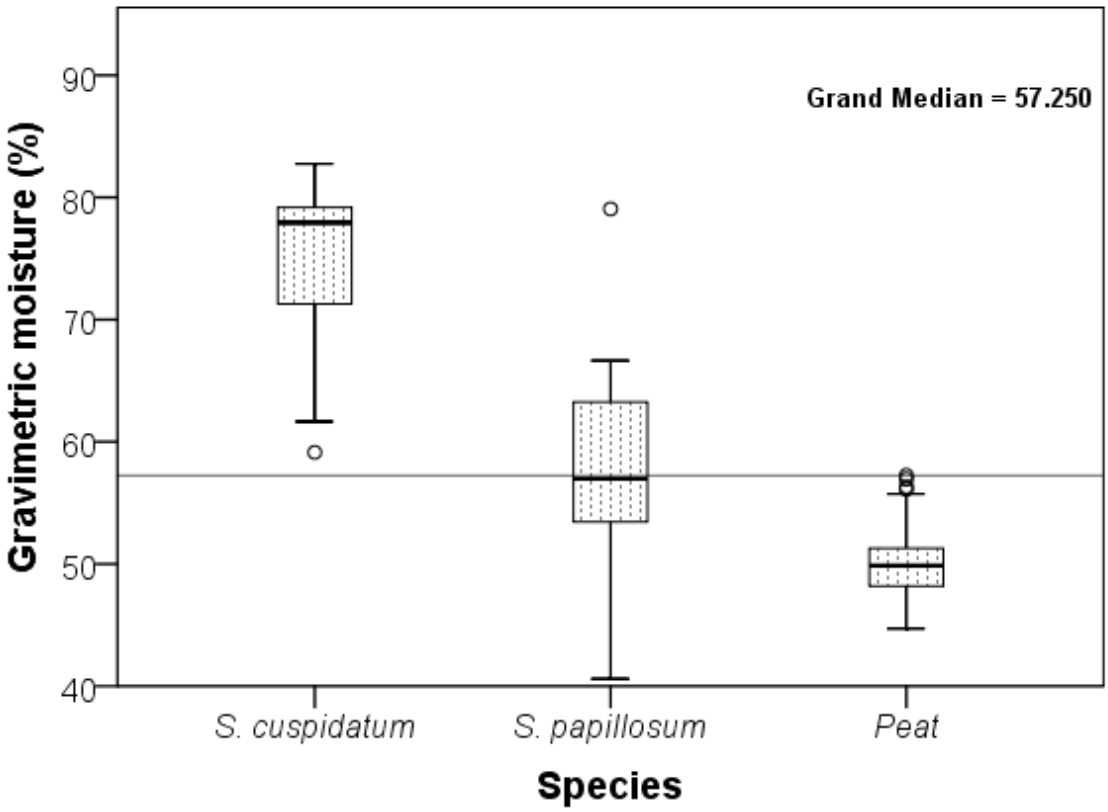

Figure 4.8. Gravimetric moisture (% water of fresh weight of sample) by the different *Sphagnum* species and peat. Data shown are from all treatments (n = 27). The box indicates the 25th percentile, the median (central line), and the 75th percentile. The line across the graph indicates the median of all the data. The whiskers show the maximum and minimum values. The dot is an outlier (< 1.5 IQR).

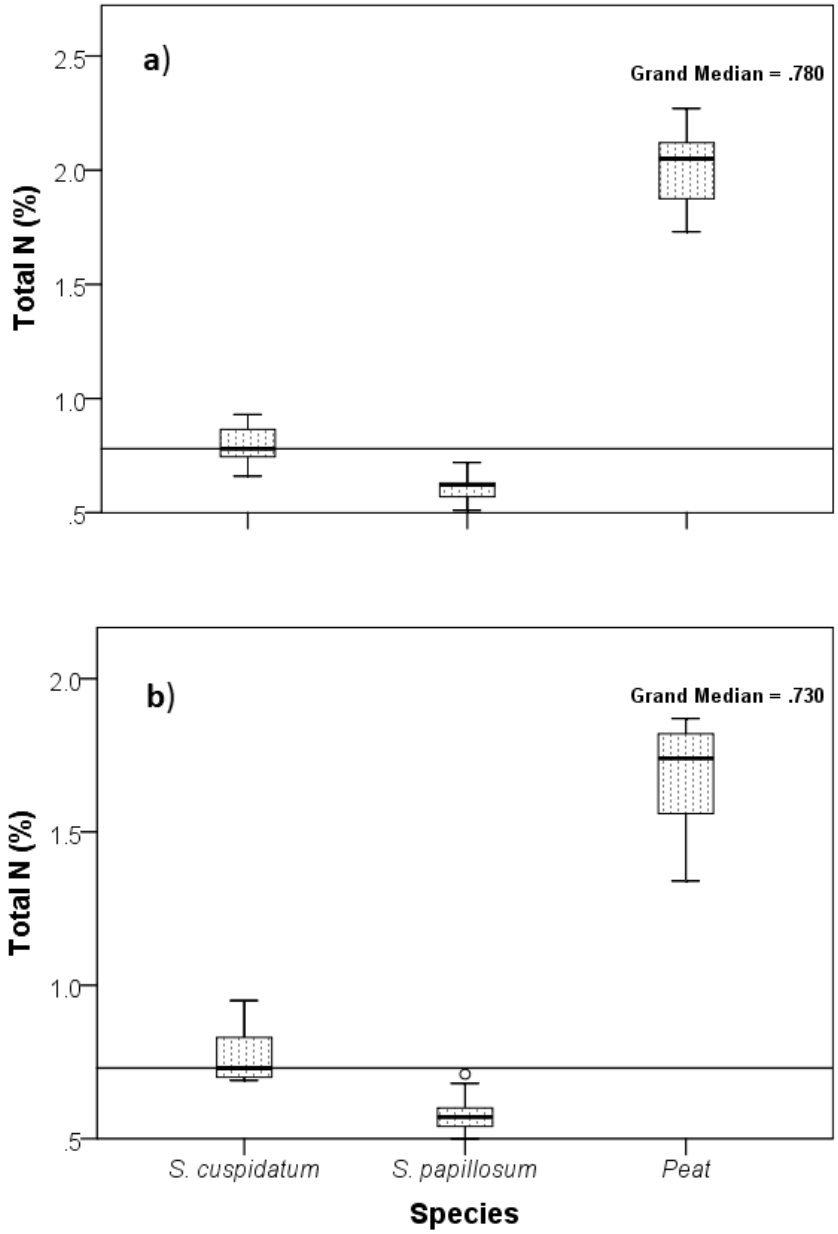

Figure 4.9. Total N (%) content in the two *Sphagnum* species and peat. Data shown in a) are from the P & K treatments (n = 15); and in b) are from Mg & MRMs treatments (n = 12). Line crossing the graphs indicates the median of all the data (from the three different species). The box indicates the 25th percentile, the median (central line), and the 75th percentile. The whiskers show the maximum and minimum values. The dot is an outlier (> 1.5 IQR).

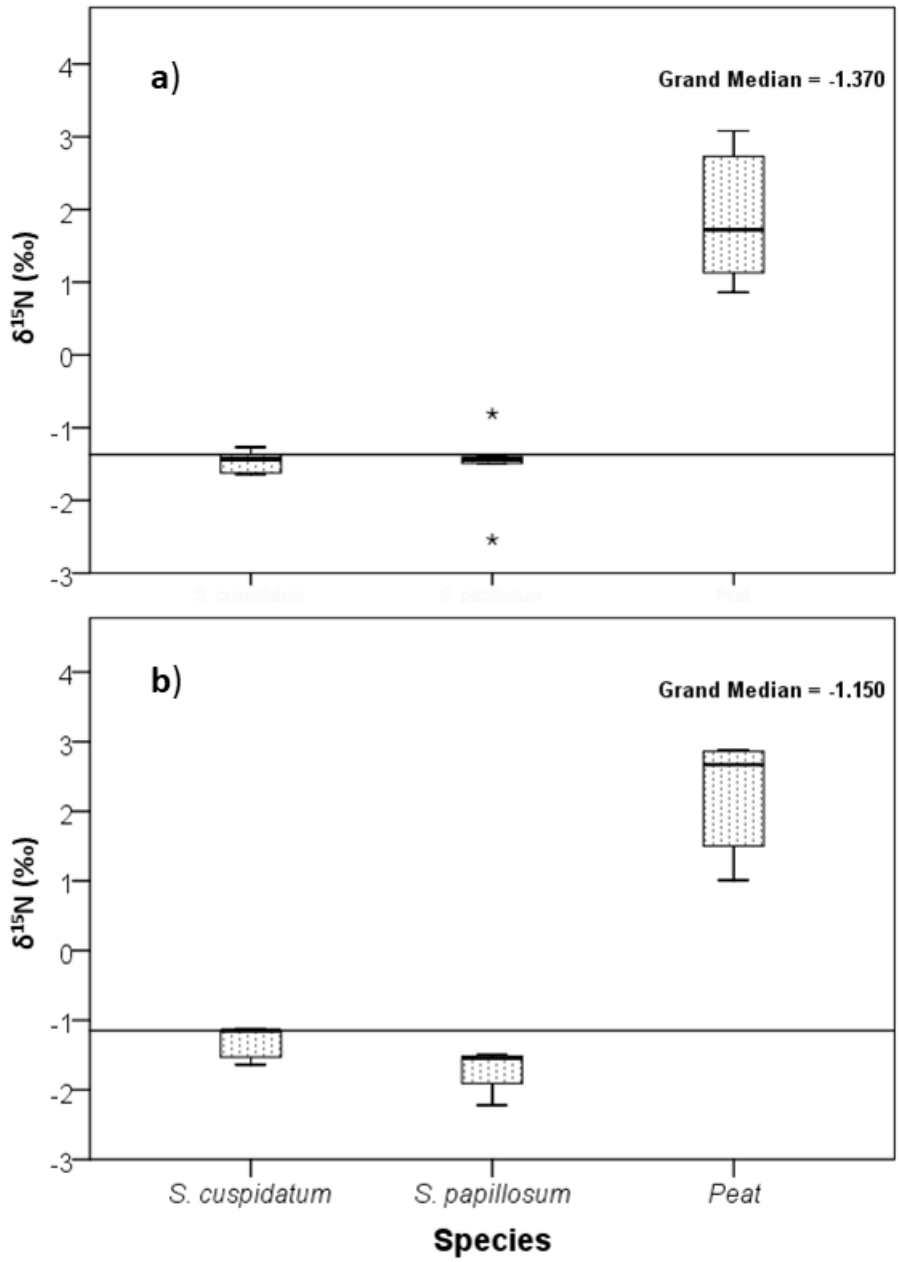

Figure 4.10. $\delta^{15}N$ (‰) signature in the two *Sphagnum* species and peat. Data in graph a) are from the P & K treatments (n = 15) and data in b) are from the Mg & MRMs treatments (n = 12). Line crossing the graphs indicates the median considering all the three species. The box indicates the 25th percentile, the median (central line), and the 75th percentile. The whiskers show the maximum and minimum values. The stars are outliers (> 3 IQR).

4.5 Discussion

These results showed that exposure to high rates of atmospheric Nr deposition and thus saturation of N, did not shut down completely BNF activity in *Sphagnum* dominated peatlands (Fig. 4.4 and Fig. 4.6). Complete shutdown has been suggested in different studies done in boreal forests (Zackrisson et al., 2004; Ackermann et al., 2012; Rousk and Michelsen, 2016). BNF rates were suppressed by ~86% when added only Nr, when Nr was added along with P&K the suppression was a little bit lower, by ~83% (~37% considering the mean instead of the median). On the other hand, it supports the idea that, in heavily polluted areas such as Central Europe (Zechmeister-Boltenstern and Kinzel, 1990) or Western Europe (van den Elzen et al., 2017) with high rates of atmospheric Nr deposition, BNF still plays an important role as source of N for the ecosystem.

The saturation of N suppressed median BNF rates by 86%; however, the addition of other nutrients as P, K, and Mg, as well as MRMs, augmented the BNF activity again. In fact, the addition of P (60.5 μg P per pot) improved the BNF rates by 37% considering the median values, which is in line with the results found in temperate regions in a study with *Sphagnum* mosses of a peatland under high rates of Nr deposition (van den Elzen et al., 2017), and in a study with grass in a restored prairie (Reed et al., 2007). In contrast, Zheng et al. (2019), in a global meta-analysis across different biomes, found a decrease in free-living BNF after P addition, mainly in temperate/boreal forests. The addition of 605.2 μg of P per pot resulted just in an increase of the BNF rates by 26% (considering the median and discarding thus an extreme value), suggesting that the lower amount of P is enough to satisfy the P needs of the bacterial community, or that there may be a limitation by a third element, K, as indicated in other studies focused on plant nutrition (Bragazza et al., 2004; Larmola et al., 2013). A study done on feather mosses found no effects or negative effects of P additions on BNF rates (Rousk et al., 2017). Elevated rates of P during long periods may also result toxic for mosses

(Bubier et al., 2007; Rousk et al., 2017). It was found that the addition of K only, 121 μg per pot, derived in a slight increase of the BNF rates by 11%, suggesting a limiting role of P under high rates of Nr deposition. The possibility of interference by extra Nr with the K due to the form of the addition (KNO_3) was considered, but under the N saturation due to high rates of Nr deposition the amount of extra Nr was less than 4% of the amount already added, and the total amounts were not significantly different. The addition of P and K combined, 605.2 and 121 μg per pot respectively, resulted in an increase of the BNF rates by 83%, confirming the co-limitation effect of P and K in peatlands exposed to N saturation, not only for *Sphagnum* growth (Bragazza et al., 2004), but in this case for BNF.

The addition of Mg (121 μg per pot), considering median values, resulted in an increase of the BNF rates by ~58%, suggesting that Mg not only plays a key role in alleviating the effects of N saturation in the plant (Bragazza et al., 2004), but also in the BNF process. The idea that was presented by Soares & Pearson (1997) was that the mosses in order to counteract the increase of hydrogen ions, due to an excess in Nr inputs (particularly NH_4^+), they mobilise nutrients such as Ca, Mg, and K, and their use in other processes could be limited. The extra P, Mg, and K may allow the mosses to fight against the excess in N at the same time that they can use them for the BNF processes. The addition of MRMs (CO_2, N_2O, CH_4 520, 1.26, 3.44 ppm respectively) induced an increase in BNF rates by 80% (median values) which is within the range of values obtained in a study with marine cyanobacteria that reported BNF rates 3 to 20 times higher with elevated CO_2 (Levitan et al., 2007), and in line but higher than the increase reported from N_2-fixing trees under elevated CO_2 as well that was by 23% (Tissue et al., 1996). On the contrary, Warren et al. (2017) found no effects on peat BNF rates by higher concentrations of CO_2 on the one hand, or higher concentrations of CH_4 on the other, however, they have not done the incubations with both gasses at the same time, and thus with the possibility of benefiting from the synergies of the interactions

among different bacterial communities such as methanotrophs, phototrophs, and autotrophs (Larmola et al., 2014). And the addition of Mg and MRMs together resulted in an increase of the BNF rates by only ~46% considering the median values, but by 88% considering mean values. The significant increase of BNF rates due the MRMs, apart from the synergies among the indicated bacterial communities, it could be favoured as well by the energy provided by N_2O reductase enzyme through N_2O reduction to N_2 and coupled this process to N_2 fixation (Farias et al., 2013; Desloover et al., 2014). These results suggest that although there may be some maximised synergies by the combined treatment in some cases (e.g. for *S. cuspidatum*), there may not be in others (e.g. in peat with incubations done in the dark and thus not affected by an increase in photosynthesis).

The BNF rates in both experiments with *Sphagnum* mosses and peat showed high variability, which is similar to other studies in the field under natural conditions (Larmola et al., 2014; Vile et al., 2014), or in the laboratory in a mesocosm experiment (van den Elzen et al., 2017). In the case of the P&K addition experiment (Fig. 4.5) although the peat median BNF rate was the highest, it was *S. cuspidatum* BNF rates the ones reaching the maximum values, that confirms that BNF rates are higher in hollows than in hummocks (Stewart et al., 2011) and thus confirming the importance of moisture in the regulation of BNF rates (Rousk et al., 2018). Moreover, looking at the BNF rates by species in the second experiment (Fig. 4.7), it can be observed that the difference in the rates obtained by *S. cuspidatum* in comparison with the other species are huge. It suggests the existence of a hot spot (McClain et al., 2003), and important differences in the microbial community between hollows and hummocks within the *Sphagnum* species (Opelt et al., 2007) as well as between *Sphagnum* species and peat. To reach such high rates of BNF by *S. cuspidatum* it could have been because of several favourable conditions. First, tissue hydration that was significantly higher with respect to the other species (Fig. 4.6), as they were semi-submerged in water, mimicking usual natural

conditions. Second, nutrient availability, particularly P&K that alleviate N excess and are essential for ATP and photosynthesis. And third, high MRMs concentrations that boosted N_2O reduction (Desloover et al., 2014), methanotrophy (Raghoebarsing et al., 2005), and photosynthesis (Weston et al., 2015), which was enhanced also due to optimum light conditions (photosynthesis in *Sphagnum* mosses reaches its maximum at PAR ~650 µmol m^{-2} s^{-1}) and therefore making available more ATP to be used in the BNF process.

These results showed a positive correlation between N content of the moss and peat species and BNF rates, similar to the ones reported in a study under high rates of Nr deposition (van den Elzen et al., 2017). They also showed a significant difference among the species regarding N content (Fig. 4.9) where *S. cuspidatum* has a higher N content than *S. papillosum*, which is similar to differences found in the hummock-hollow complexes in arctic ecosystems (Stewart et al., 2011) that demonstrate that hollow *Sphagnum* spp have a better performance because of higher moisture content and access to mineral nutrients than hummock ones (Robroek et al., 2007). *Sphagnum* mosses are adapted to N-poor environments, and they have developed a mechanism for rapid N uptake when it is available (e.g. rain event) as defence against vascular plants in peatlands, however, under a longer period (days) of N availability the N uptake rates decrease as temporal storage pools such as cell walls and vacuoles get saturated, and they continue at the rate of N consumption through amino acid and protein synthesis and biomass and chlorophyll production (Fritz et al., 2014). The continuity of the nitrogenase activity, even though the system was saturated with Nr, may have been due to the luxury consumption of N and its assimilation by the *Sphagnum* mosses that was enhanced by cell water content, and availability of P and CO_2 (Limpens and Berendse, 2003). On the other hand, high rates of Nr deposition have been associated with a decrease in *Sphagnum* moss growth, and thus with lower N consumption rates, and also with a stagnation of photosynthesis even with increasing the chlorophyll levels, but this effects

were found in experiments after years of high rates of Nr fertilization (Wiedermann et al., 2009; Fritz et al., 2012). In the short term, under no N limitation, plant growth can be limited by other nutrients availability such as P, K, and Mg (Bragazza et al., 2004) and by CO_2 availability (Limpens and Berendse, 2003), limitations that also affect BNF as these results have shown.

BNF activity under high rates of Nr deposition, this is N saturation, is also confirmed by the $\delta^{15}N$ signature in *Sphagnum* mosses and peat (Fig. 4.10). After seventeen days of N background treatment (total amount applied of 1091.25 µg N per pot), and ten days of additional treatments, the $\delta^{15}N$ signature of the *Sphagnum* mosses and peat did vary, but not significantly, with respect to the values obtained in the field (Forsinard, Scotland) under natural conditions. Values close to 0‰ indicate that BNF plays an important role in supplying N (Stewart et al., 2011). The $\delta^{15}N$ values of peat were positive (median of 2.15‰), suggesting that BNF and Nr deposition are the main sources of N; while the ones of the *Sphagnum* species (*S. cuspidatum* and *S. papillosum*) were negative (median of -1.41‰) and closer to 0‰ indicating that BNF played a more important role. This difference between the $\delta^{15}N$ values of peat and *Sphagnum* species was significant. These results were in line with the $\delta^{15}N$ values obtained in *Sphagnum* mosses and peat of other peatlands (Knorr et al., 2015; Zivkovic et al., 2017; Novak et al., 2019).

It can be concluded that although there was a significant decrease in the BNF rates under Nr saturation, i.e. the addition of 1091 µg N per pot, BNF activity did not shut down completely.

The addition of P resulted in higher BNF rates. The addition of K resulted in just a slight increase in BNF rates, demonstrating the co-limitation with other elements. And the addition of the combination of P&K led to a significant increase in the BNF rates (83%), confirming the co-limiting role of P & K in BNF activity when N is no longer scarce.

Once N, P, and K are not limiting nutrients there are other elements that come into play regarding BNF activity such as Mg that is essential for BNF and it is used by the moss to avoid intoxication with N so its addition stimulated BNF rates by 58%; and MRMs that stimulated BNF rates by 80%, suggesting that energy generated by N_2O reduction, methanotrophy (fuelled by CH_4), and photosynthesis (fuelled by CO_2) is critical in boosting BNF and thus that it may work as a limiting factor.

The lack of nutrient (P, K, and Mg) and energy (MRMs) limitation under N saturation resulted in a pattern of maximum incorporation of N (N uptake and assimilation) linked to a maximum of BNF activity. These results were just short-term response of BNF to specific conditions measured, in the gasses case, through short term incubations. Therefore, long term nutrient fertilization treatments would be needed to evaluate BNF response as well as if this fertilization would eventually support vascular plants to take over mosses. It also would be needed long term incubations to evaluate BNF response to MRM gasses.

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CHAPTER 5: EFFECTS OF ELEVATED ATMOSPHERIC CO_2 ON BIOLOGICAL NITROGEN FIXATION IN A TEMPERATE FOREST

5.1 Abstract

Since the beginning of the past century, there has been a massive increase in CO_2 in the atmosphere, and it is expected to continue growing in the decades to come. This increase in carbon dioxide availability can enhance net primary production (NPP) and thus C sequestration, reducing the concentration of CO_2 in the atmosphere. However, NPP increase could be limited by the availability of nitrogen (N) to trees, which is the main limiting factor for plant growth in many environments. The effects of elevated CO_2 on biological nitrogen fixation (BNF) in mature temperate forests are poorly understood. It was hypothesized that the NPP increase under elevated CO_2 will increase N demand and thus BNF activity. To test if BNF was affected by elevated CO_2 BNF was measured in epiphytic feather moss *Hypnum cupressiforme* samples and in soil (top 0 – 5 cm) samples using the direct $^{15}N_2$ assimilation method under elevated and ambient CO_2 conditions in the BiFOR Free-Air Carbon Dioxide Enrichment (FACE) facility. It was also measured C and N content, main macro and micronutrients, gravimetric moisture, and $\delta^{15}N$ natural abundance in soil and bryophyte samples. It was found that the BNF in *H. cupressiforme* differed under elevated

CO_2 and under ambient CO_2 but not significantly. The $\delta^{15}N$ signature was also lower in the former than in the latter, but still with very low values, ranging between -8‰ under elevated CO_2 and -7‰ under ambient CO_2, suggesting that the main source of N comes from the agricultural land that surrounds the forest through atmospheric reactive nitrogen (Nr) deposition (23 Kg N ha^{-1} yr^{-1}). The C:N ratio did not vary under elevated and ambient CO_2 conditions. Contrarily, BNF in soil samples (0-5 cm top soil) were 369% higher under elevated CO_2 than under ambient CO_2, with a $\delta^{15}N$ signature close to 0‰, and significant differences in the C and N content and the C:N ratio, being higher under elevated than under ambient CO_2 conditions. It was also found that the concentrations of Mg, K, Co and Ni in soil were lower under elevated CO_2, and higher BNF rates, than under ambient CO_2. These results suggest that elevated CO_2 would not only imply an increase in NPP but also an increase in BNF that may partially satisfy a higher N demand.

5.2 Introduction

Since the Industrial Revolution in the 18th century, there has been a massive increase in the concentration of CO_2 and reactive nitrogen (Nr) in the atmosphere due to human activities, not only by increasing emissions (e.g. increase of fossil fuel burning) but also by eliminating natural sinks (e.g. deforestation, wetlands desiccation). Regarding CO_2, the concentration has increased from ~280 ppm in 1750 (Team et al., 2014) to 411 ppm in 2019 (Dlugokencky and Tans, 2019), in fact, the increase rate in the first decade of the 21st century has been the fastest in history at a pace of 2 ppm per year (Team et al., 2014). At this rate, by 2100 the concentration of CO_2 in the atmosphere will be about 570 ppm; however, if the measures taken for the reduction of GHG emissions (negative emissions technologies – NETs) are effective it is expected the concentration to be lower, many scenarios showing a range between 430 – 480 ppm (Smith et al., 2016). In the case of atmospheric Nr deposition, the global deposition rate was 3 times higher in 2016 than in 1850 and was estimated between 125 and 132 TgN per year (Kanakidou et al., 2016), however it has a significant difference with CO_2, which is that its effects are more localised (Llado et al., 2017).

Forests are a C sink, in fact, they stored about 67% of the terrestrial C (Llado et al., 2017). Plant growth is limited by CO_2 as key ingredient of photosynthesis (Finzi et al., 2002), so the predictions of a progressive increase of atmospheric CO_2 concentrations may result in higher rates of plant growth and net primary productivity (NPP), i.e. the amount of energy trapped less that lost by respiration (Zak et al., 2003; McCarthy et al., 2010; Hungate et al., 2013; Talhelm et al., 2014). Many experiments that have been carried out in forests with elevated CO_2 fumigation have shown an increase in NPP in the long-term (Norby et al., 2005; Zak et al., 2011). However, some studies have found that, under elevated CO_2, after initial higher rates of NPP and therefore higher rates of nutrient consumption, a decrease in nutrient availability (e.g. in N availability known as progressive nitrogen limitation - PNL)

may cause a decline in NPP (Zak et al., 2003; Luo et al., 2004; Finzi et al., 2006). In addition, in a forest limited by P, it has been found that an increase in CO_2 increases photosynthesis but did not increase tree growth or NPP (Hasegawa et al., 2018).

In forest ecosystems, N plays a key role as a limiting factor for tree growth (Binkley and Hogberg, 1997). The researchers that have developed the PNL hypothesis have proposed two main routes to explain why it happens: one is related to the biological principle that to create biomass N is needed which would be immobilised and therefore the soil N pool would decrease slowing N-mineralization rates; the other is that CO_2 availability would enhance microbial activity that would imply a higher N demand that would result in immobilisation (Luo et al., 2004; Finzi et al., 2006). On the other hand, some studies have found no N limitation after more than a decade of elevated CO_2 suggesting that the surplus of N was obtained because of the increased availability of C belowground that stimulated microbial activity, increasing the decomposition rates that were combined with a higher fine root production for better nutrient uptake (Finzi et al., 2007; Drake et al., 2011). Little has been done to study the contribution of BNF into the soil Nr pool under elevated CO_2, and some of the studies that considered this N source indicated that it was negligible (Drake et al., 2011; Hofmockel et al., 2011), while Terrer et al. (2016) in a modelling study demonstrated that BNF is a key contributor to NPP in forests under elevated CO_2.

Studies done on BNF have presented contrasting results regarding the N contribution of this process to the N cycle in forests exposed to elevated CO_2. A study focused on heterotrophic N_2 fixation in a pine forest indicated that CO_2 did not affect BNF and thus that there was no additional contribution of N (Hofmockel and Schlesinger, 2007), which was similar to the results found in an oak woodland that indicated that nonsymbiotic BNF was unaffected by elevated CO_2 (Hungate et al., 2014). However, other studies carried out in wet soils have found higher BNF rates under elevated CO_2, e.g. in rice soil (Cheng et al., 2001), or wetlands

(Dakora and Drake, 2000). Another study focused on symbiotic BNF in a scrub-oak indicated that the first four years BNF rates were higher under elevated CO_2, but the next three years BNF rates declined (Hungate et al., 2004). A study done with young tree species (6 years old) in a mixture between fixing and non-N_2-fixing species reported that BNF rates did increase for all trees growing in mixture under elevated CO_2 (Millet et al., 2012). So far, no published studies to date have assessed the effects of elevated CO_2 on BNF in temperate mature deciduous forests. There is also no evidence of the effects of elevated CO_2 on BNF in epiphytic mosses (i.e. mosses growing on tree boles). However, it is known that epiphytic mosses can host N_2-fixing organisms (DeLuca et al., 2002; Jean et al., 2012; Rousk et al., 2018), and thus they can be an important source of N for the ecosystem as it has been found in tropical forests (e.g. Coxson, 1991; Han et al., 2010), temperate forests (e.g. Lindo et al., 2011; Jean et al., 2012), and boreal forests (e.g. DeLuca et al., 2002; Zackrisson et al., 2009). Therefore, it is not clear whether N availability and enhanced NPP under elevated CO_2 in temperate forests will prime up microbes for N fixation to meet plant N demands.

In this study the effects of elevated atmospheric CO_2 fumigation on BNF were investigated in a newly establish FACE experiment, a temperate mature forest in Staffordshire, under the Birmingham Institute of Forest Research. It was hypothesized that elevated CO_2 fumigation of forest increases BNF rates because there is a higher N demand by plants. To investigate this, BNF rates were measured in soil samples from the forest floor (0 – 5 cm; O horizon), and in *Hypnum cupressiforme*, an epiphytic byrophytic moss that was growing on the bark of the trees, using the $^{15}N_2$ assimilation method (Saiz et al. 2019). Additionally, in order to assess other factors that could affect BNF, it was also measured gravimetric moisture and the main macro and micronutrients contents of soil and the bryophytes. C and N content was determined in the samples, as well as C:N ratio, and the natural abundance of $\delta^{15}N$.

5.3 Material and methods

5.3.1 Study site

The Birmingham Institute of Forest Research (BiFOR) free-air CO_2 enrichment (FACE) experiment (see Hart et al., 2019) was located in Staffordshire, UK (52º80' N, 2º30' W). The forest covers an extension of more than 19 ha and the height ranges from 92 m.a.s.l. at its lowest point to 112 m.a.s.l. at its highest (Hart et al., 2019). It is a mature deciduous temperate woodland (~150 years old), that is dominated by English oak (*Quercus robur*) and hazel (*Corylus avellane*) coppice, in addition to other species such as silver birch (*Betula pendula*), holly (*Ilex aquifolium*), and sycamore (*Acer pseudoplatanus*). The ground vegetation is almost testimonial, with a lot of deadwood present in all its forms (standing, falling, woody debris; Fig 5.1). The mean annual temperature for 2017, measured at 23 m height, was 10.3 ºC, and the mean annual precipitation in the same period was 624 mm (Hart et al., 2019), and the number of days of the growing season was 275 (Norby et al., 2016). The atmospheric reactive nitrogen deposition rate was on average (2014-16) of 23 kg N ha^{-1} yr^{-1} (Air Pollution Information System). The experiment consisted of 3 infrastructure rings fumigated with CO_2 (150 ppm of CO_2 in addition to ambient CO_2, total concentration of ~550 ppm) and 3 infrastructure rings with no CO_2 fumigation that are the controls (it is added 0 ppm of CO_2 to the ambient concentration of about 400ppm), and there are also 3 gost areas (no rings) where there is no infrastructure (Fig. 5.2). The rings are 30 meters in diameter and between 24.7 and 27.3 m in height because of being about 1 m above the canopy, so it varies among rings (Fig. 5.3). The fumigation operation lasts about 18 hours per day, between 5 am and 10 pm; and it is carried out during the growing season, so approximately between April and October. The fumigation with CO_2 started in April 2017 and it is expected to run for at least ten years.

Figure 5.1. View of the ground and understory vegetation of one of the rings at BiFOR (FACE) facility.

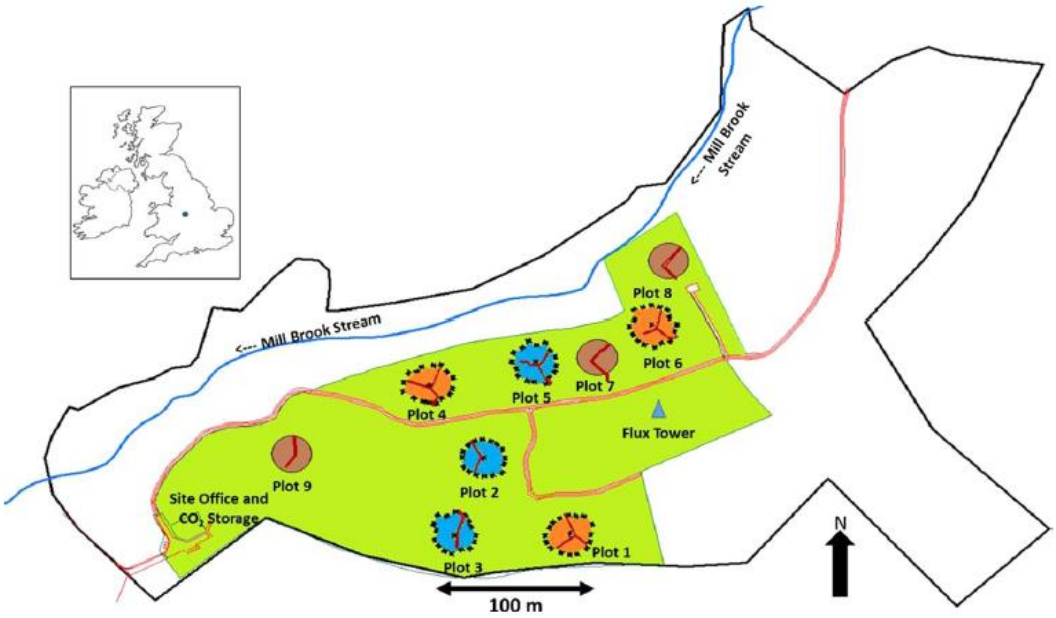

Figure 5.2. BiFOR FACE facility schematic map. Fumigated rings are in orange (plots 1, 4, and 6). Non-fumigated rings are in blue (plots 2, 3, and 5). No infrastructure rings are in brown (plots 7, 8, and 9). Within the rings the lines in red represent elevated walkways. Green area is the experimental area controlled by the University of Birmingham (7.3 ha). The black outer line represents the forest that extends 19.1 ha. The Inset map indicates the location in the UK. (Source: Hart et al., 2019)

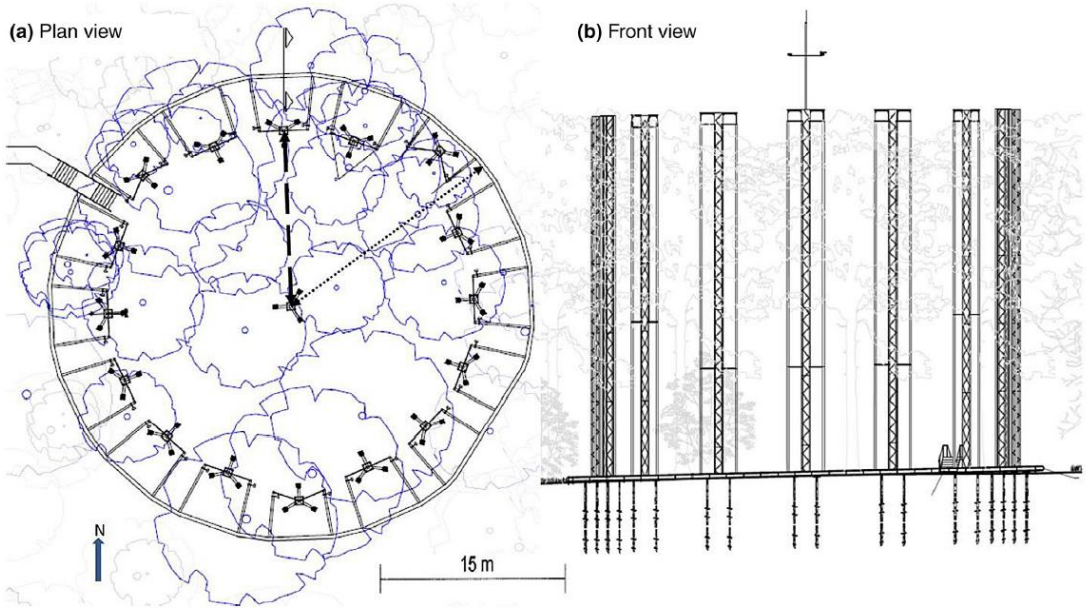

Figure 5.3. Schematic example of an infrastructure ring at BiFOR FACE. a) Plan view of the 30 m in diameter experimental ring. Dotted arrow indicates radius from the centre to outer plenum edge (R = 20 m) and the dashed arrow shows the radius from the centre to the inner edge of the ring (R = 15 m). Note that the towers are not distributed uniformally which was due to existing trees that was necessary to avoid. b) Front view showing screw pile system penetrating into the bedrock, so that it was not necessary external cables to support the towers. (Source: Hart et al., 2019)

5.3.2 Sampling approach

During the beginning of the growing season in May 2018, 5 random samples of forest soil (top 5 cm, O_i horizon) were collected in each of the 6 rings, 3 with elevated CO_2, and 3 control (ambient CO_2) that were passed through a 2 mm sieve to discard the big pieces of woody debris. Two weeks later, under a heatwave and therefore dry conditions, epiphytic mosses (*Hypnum cupressiforme*) that were growing on the bottom part of live tree trunks were also collected. It was taken a bunch of mosses from each of the closest trees where the 5 soil samples where collected in each ring under elevated CO_2 and control. So, five

replicates of mosses per ring were taken. Each replicate consisted of more than 20 shoots, enough to fill around half of the 50 ml serum bottles for incubation.

5.3.3 $^{15}N_2$ direct assimilation method

At each ring, four replicates were incubated with $^{15}N_2$ gas (98 atom% Cambridge Isotope Laboratories Inc., USA) while the fifth was used as control (incubated using just air). Each replicate consisted of 10 g of forest soil or 25-30 shoots of the moss *H. cupressiforme*. The procedure is explained in depth elsewhere (Saiz et al., 2019), so here it is going to be explained briefly. The samples (moss or soil) were placed in 50 ml serum vials that were capped with gas-tight rubber septa. After closing the vials, 5 ml of the headspace air were evacuated and replaced by the same amount of $^{15}N_2$ (98 atom%) gas, being approximately 10% headspace concentration, and they were located in a similar place were the samples were collected: in the case of the soil, under some leaf and woody litter; and in the case of *H. cupressiforme* close to a trunk. They were incubated for 24 hours, avoiding thus long-term incubation problems such as oxygen depletion (Myrold et al., 1999).

After the 24th hour, the incubation was stopped, bottles were aerated to remove any residual $^{15}N_2$ gas, and the samples were transported, immediately, to the laboratory in a cool box with ice. The soil and bryophytes were weighed, dried in the oven at 70 ºC for 72 hours, and weighed again so that gravimetric moisture could be calculated (% of water). Then the samples were milled and pulverised manually with a mortar and subsamples were sent to the Life Sciences Mass Spectrometry Facility at the Centre for Ecology and Hydrology, Lancaster for ^{15}N content analysis by Isotope Ratio Mass Spectrometry (IRMS) using a Carlo Erba NA1500 elemental analyser (Italy), coupled to a Dennis Leigh Technologies isotope

ratio mass spectrometer (UK). The analytical precision of the instrument was 0.36‰. The BNF rates were calculated using the following formula (Equation 5-1; Liengen, 1999):

$$Y = \left(\frac{atom\%\ ^{15}Nexcess}{100}\right) x \left(\frac{totalNsample\ x\ 10^9}{t\ x\ 28}\right) x\ \left(\frac{100}{\%^{15}N\ air}\right)$$

where Y (nmol N gdw^{-1} h^{-1}) is the amount of N_2 fixed during the experiment, atom% $^{15}Nexcess$ is the difference between $atom\%^{15}Nsample$ and $atom\%^{15}Ncontrol$, total N is the total amount of nitrogen in the sample (g N 100 gdw^{-1}), t is the incubation time, 28 is the molecular weight of N_2 (g/mol), and $\%^{15}Nair$ is the percentage of ^{15}N out of the total amount of N gas in each incubation vial.

5.3.4 Analysis of selected elements

Subsamples of forest soil and *H. cupressiforme* were used to determine their amount of carbon and nitrogen content. They were sent to the laboratory of the School of Geographical Sciences of the University of Bristol. They were analysed using a Thermo Scientific FlashEA 1112 Nitrogen and Carbon analyser. The instrument had a limit of detection for both C and N of 0.01%, and the precision was determined by repeated analysis of a soil reference standard (0.21% N and 2.39% C) and the relative standard deviation (RSD) was below 5%.

Forest soil and *H. cupressiforme* pulverised subsamples of 0.2 g were digested with 8 ml HNO_3 (trace analysis grade >68%) and 2 ml H_2O_2 (30-32%, w/w, for trace metal analysis) using a MARS 6 (CEM, NC, USA) microwave digestion system. The program used was a modification of the EPA 3052 that consisted of 10 min to reach 180 ºC, and 15 min at this temperature prior to the cooling period. Then the samples were diluted 1:50 with deionised

water and filtered (0.45 µm syringe filters) in order to be analysed for Mg, K, Ca, V, Mn, Co, Ni, Cu, Mo, and P using an inductively coupled plasma mass spectrometry (ICP-MS) instrument PerkinElmer NexION 300D (Waltham, MA, USA). Table 5.1 shows the operating setup for the instrument. To determine the values from the given intensities by the ICP-MS it was generated an 8-point calibration graph from the dilution of a certified multi-component standard (VWR, UK). Every 9 samples a blank was included, and every 20, a quality control. The results were blank corrected. On average, the RSD for the ICP-MS analyses of all elements was below 4%, and the LOD was 0.1 µg/g for V, Mn, Co, and Cu; and 0.3 µg/g for Mg, K, Ca, Ni, Mo, and P.

Table 5.1. Operating setup for ICP-MS instrument.

Nebuliser Gas Flow	1.14 L/min
Auxiliary Gas Flow	1.2 L/min
Plasma Gas Flow (Ar)	18 L/min
ICP-RF Power	1600 watts
Wash solution	1% Nitric Acid

5.3.5 Statistical analysis

All data were tested for normality and homogeneity of variance using Shapiro-Wilk test and Levene's test respectively. To see the effects of elevated CO_2 into different aspects such as the BNF rates, the C:N ratio (related with the carbon content), the selected elements content, and the $\delta^{15}N$ natural abundance it was used the *t*-test. To see the correlation between BNF rates and the C:N ratio, the selected elements content, and the gravimetric moisture the Pearson Correlation Coefficient was used. Additionally, linear regression analysis was done to see the relationship between BNF rates and gravimetric moisture. Statistical analyses were performed using the IBM SPSS Statistics program, version 24. When $P < 0.05$ was obtained it was considered that there were statistically significant differences.

5.4 Results

Forest soil BNF rates were significantly higher (P = 0.011) under elevated CO_2 than under ambient conditions, in fact, a 368.7% more (Fig. 5.4a). On the other hand, the moss *H. cupressiforme* BNF rates were not significantly different (P > 0.05) between elevated CO_2 and control ring soils and they were a 59.8% lower under elevated CO_2 than under ambient conditions (Fig. 5.4b).

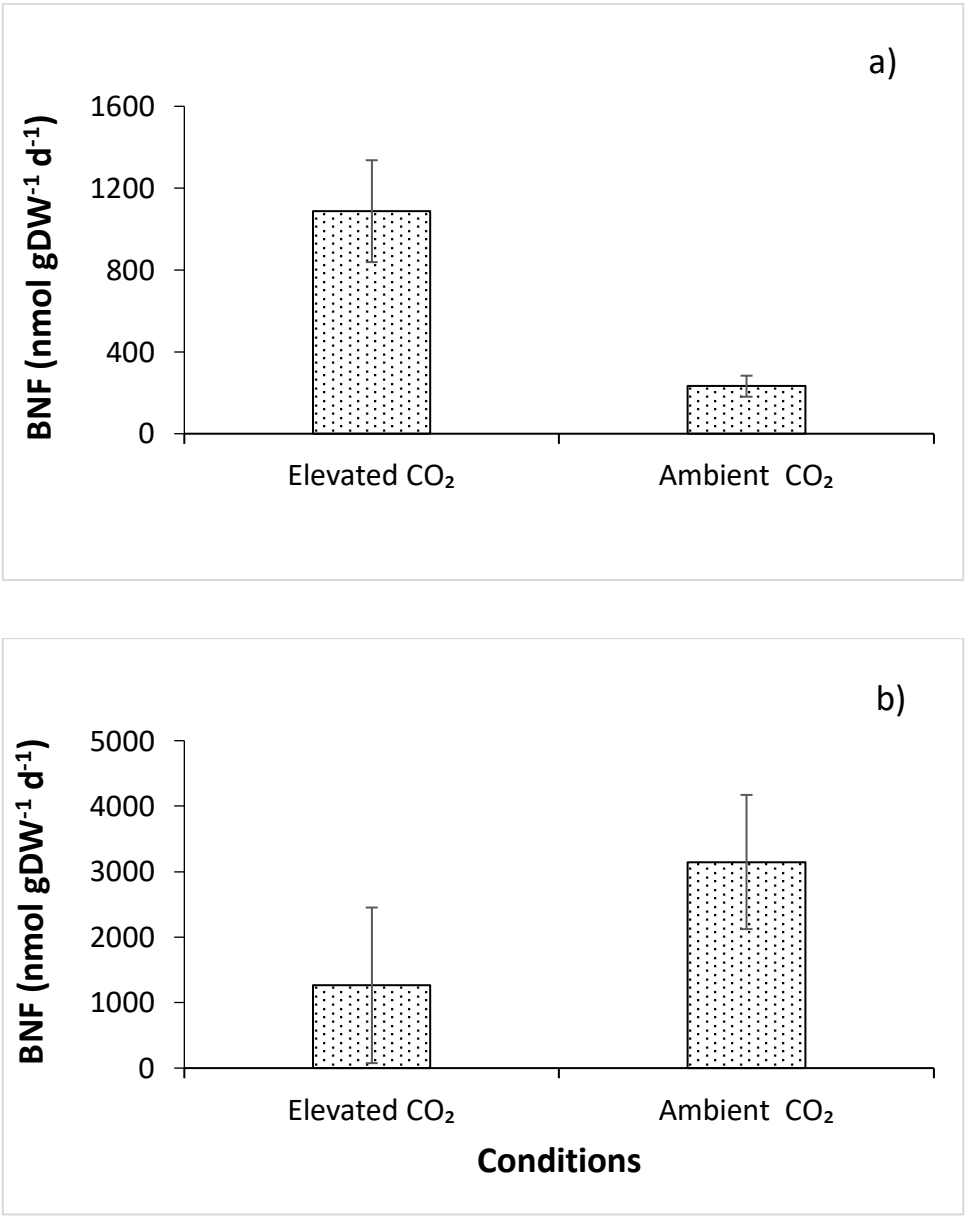

Figure 5.4. BNF rates (nmol gDW^{-1} d^{-1}) of the a) forest soil samples and b) *H. cupressiforme* under elevated CO_2 (550 ppm) and ambient CO_2. Data shown are means (±SE), n = 12.

The C:N ratio of the forest soil samples in 2018 was on average a 13.2% higher under elevated CO_2 than under ambient conditions (Fig. 5.5a), difference that was significant (P = 0.011). However, in 2019 the difference was not significant (P > 0.05) and it was lower under elevated CO_2 by 1.5% (Fig. 5.5b). The amount of C in the soil samples of 2018 under elevated CO_2 (on average 17.3%) was more than double the value under ambient CO_2 (on average 7.8%) while the amount of N under elevated CO_2 (mean of 0.86%) was almost double the value under ambient conditions (mean of 0.46%). In 2019 soil samples, the amount of C was 5.4% and 4.2% under elevated and ambient CO_2 respectively, and the amount of N 0.33% and 0.26%. In the case of the *H. cupressiforme* the C:N ratio was just a 3.3% lower under elevated CO_2 than under ambient CO_2 (Fig. 5.5c) and there were no significant differences (P > 0.05). The amount of C and N in *H. cupressiforme* was quite similar under elevated and ambient CO_2 conditions being 43.3% and 43.9% respectively for the former, and 2.02% and 2.03% respectively for the latter.

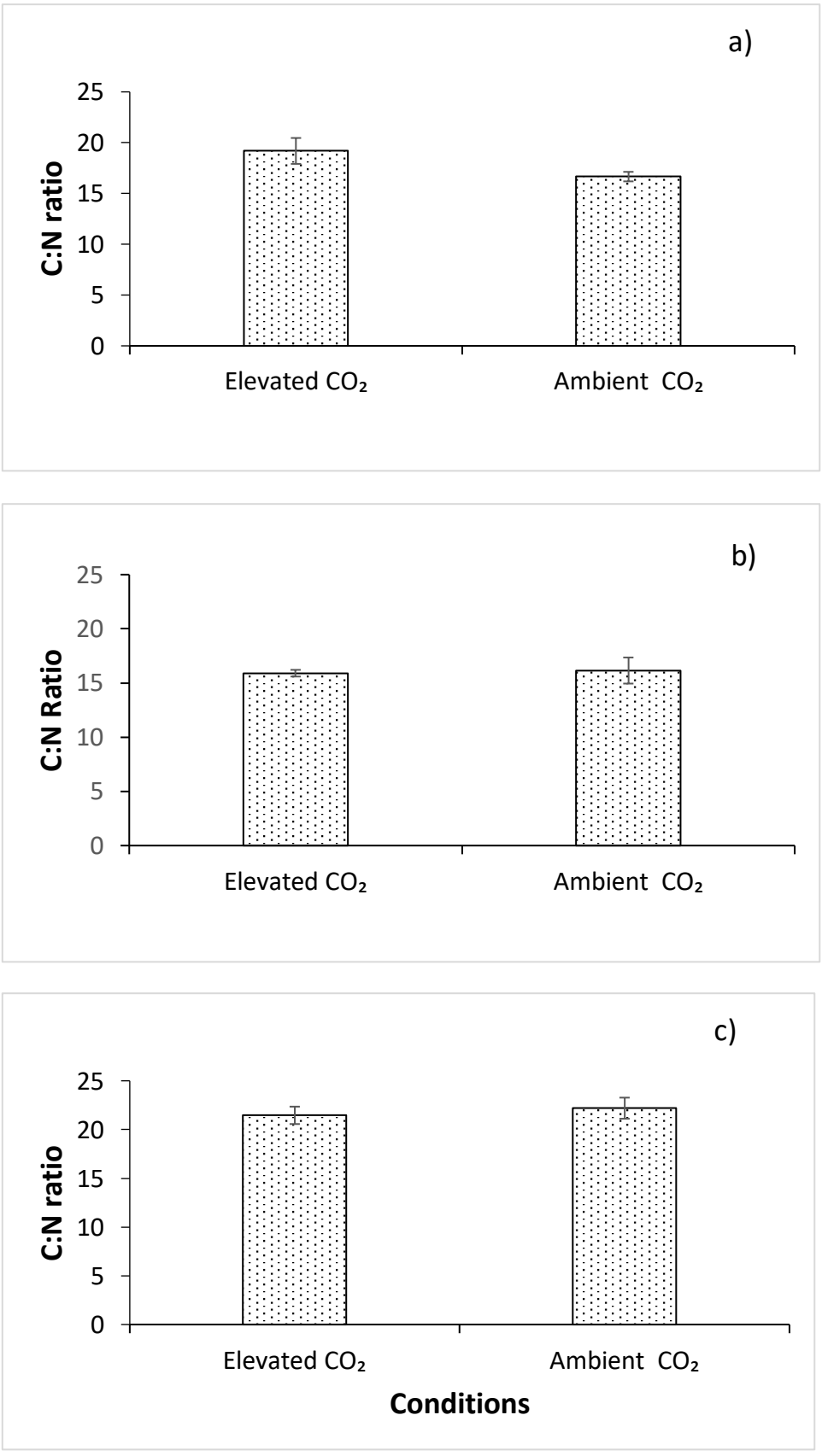

Figure 5.5. C:N ratio of the a) forest soil samples 2018, b) forest soil samples 2019, and c) *H. cupressiforme* under elevated CO_2 (550 ppm) and ambient CO_2 conditions. Data shown are means (± SE), n = 15.

There was no significant difference between CO_2 conditions (elevated and ambient) regarding the concentration of V, Mn, Cu, Mo, and P in the forest soil samples (Table 5.2); but in the case of Mg, K, Co, and Ni the concentration was significantly lower under elevated CO_2 ($P < 0.05$), while in the case of Ca, the concentration was significantly higher under elevated CO_2. In the *H. cupressiforme* samples, there were no significant differences between treatments in the concentrations of the elements studied except for K in which the concentration was higher under the elevated CO_2 than under ambient CO_2 conditions.

Table 5.2. Concentration of the selected nutrients and micro-nutrients in the forest soil samples and the *H. cupressiforme* samples under elevated CO_2 (550 ppm) and ambient CO_2 (control) in µg/g. Data shown are means ± standard error (SE). CO_2 conditions (elevated or ambient) with different letters are significantly different.

	Forest soil		*H. cupressiforme*	
Units	µg/g ±SE		µg/g ±SE	
Element	Elevated CO_2	Ambient CO_2	Elevated CO_2	Ambient CO_2
Mg	946.5 (±86.8) a	1525.9 (±91.8) b	1029.3 (±136.7) a	834.7 (±46.4) a
K	1398.1 (±65.9) a	1645.3 (±68.3) b	3330.1 (±190.8) a	2863.9 (±104.9) b
Ca	586.8 (±124.4) a	74.5 (±16.9) b	946.2 (±41.7)	975.9 (±122.5) a
V	62.5 (±4.7) a	57.7 (±0.7) a	38.3 (±1.5)	37.2 (±0.5) a
Mn	168.6 (±15.5) a	186.6 (±17.2) a	314.3 (±43.0)	330.6 (±23.2) a
Co	23.3 (±0.2) a	24.3 (±0.1) b	22.1 (±0.2)	22.2 (±0.1) a
Ni	19.9 (±0.2) a	21.6 (±0.4) b	16.9 (±0.7)	16.7 (±0.3) a
Cu	85.0 (±0.8) a	84.4 (±0.5) a	78.9 (±0.7)	79.6 (±0.5) a
Mo	56.9 (±9.3) a	37.1 (±1.0) a	38.7 (±2.6)	36.1 (±0.8) a
P	539.4 (±42.0) a	437.1 (±33.1) a	1329.3 (±48.1)	1290.1 (±90.8) a

In the forest soil samples, a negative correlation between BNF rates and the concentration of Mg and Co, and a positive one with Mn were found. Additionally, it was detected a positive correlation between BNF rates and C:N ratio. Considering the *H. cupressiforme*

samples, there was a positive correlation between the BNF rates and the gravimetric moisture (Fig 5.6). And there was a significant difference ($P < 0.001$) between species (soil and moss) regarding the gravimetric moisture (Fig. 5.7).

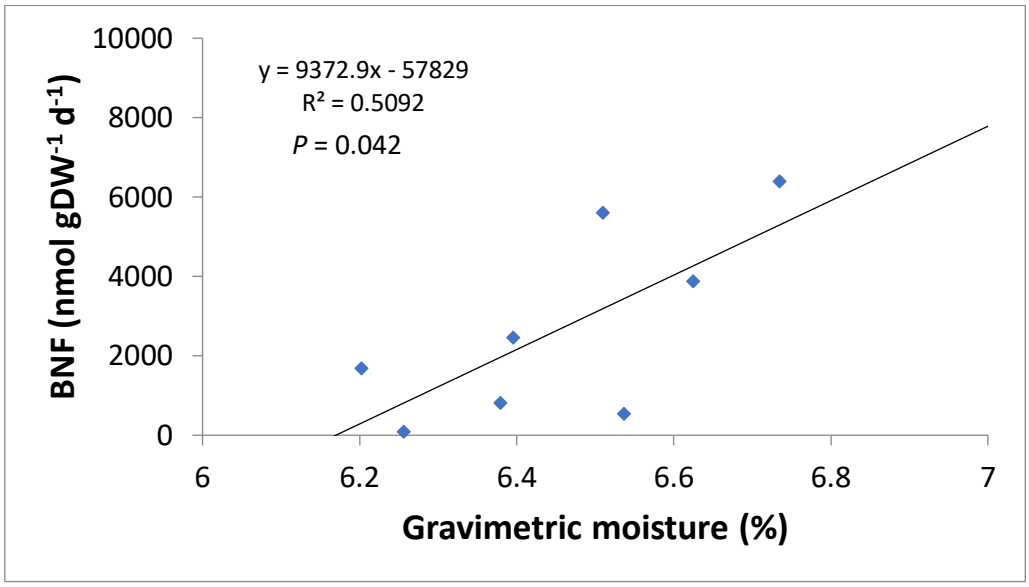

Figure 5.6. Variation of the BNF rates (nmol gDW^{-1} d^{-1}) of *H. cupressiforme* with gravimetric moisture (% of water). The line resulted from the linear regression.

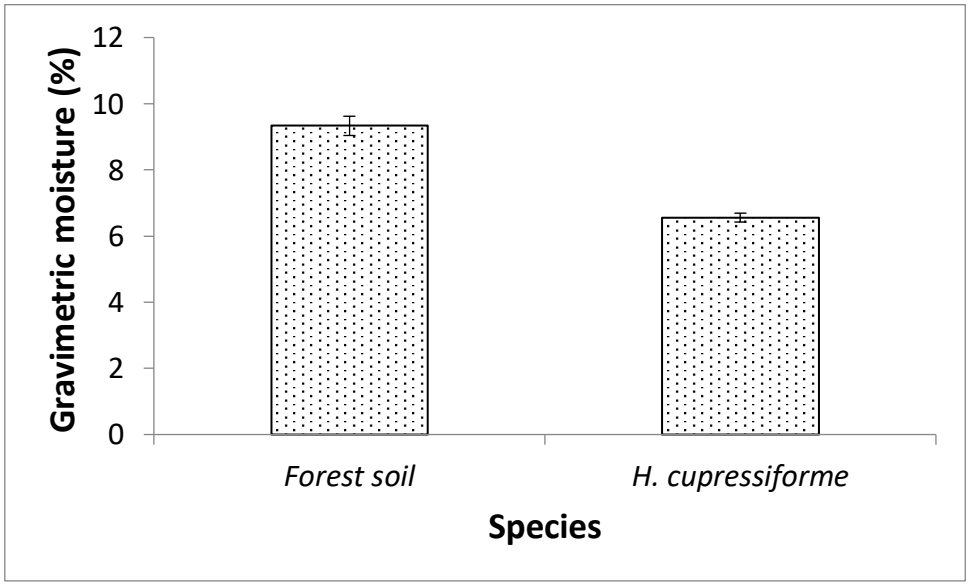

Figure 5.7. Gravimetric moisture by the two different species: forest soil and *H. cupressiforme* moss (n = 30). Data shown is % of water ±SE.

The natural abundance of the $\delta^{15}N$ signature was significantly different between the forest soil samples and the *H. cupressioforme* samples ($P < 0.001$; Fig. 5.8). The $\delta^{15}N$ of *H. cupressiforme* was significantly lower under elevated CO_2 than under ambient CO_2 (Fig. 5.9b). The $\delta^{15}N$ of soil samples, although was lower under elevated CO_2, it was not significant ($P > 0.05$; Fig. 5.9a).

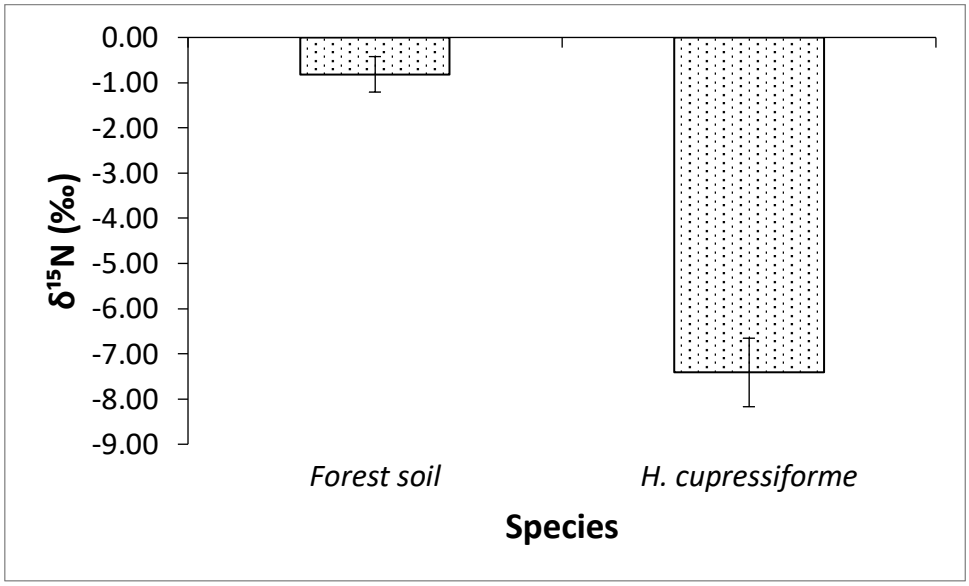

Figure 5.8. $\delta^{15}N$ natural abundance (in ‰) by species: forest soil and *H. cupressiforme* (n = 6). Data shown are means ±SE.

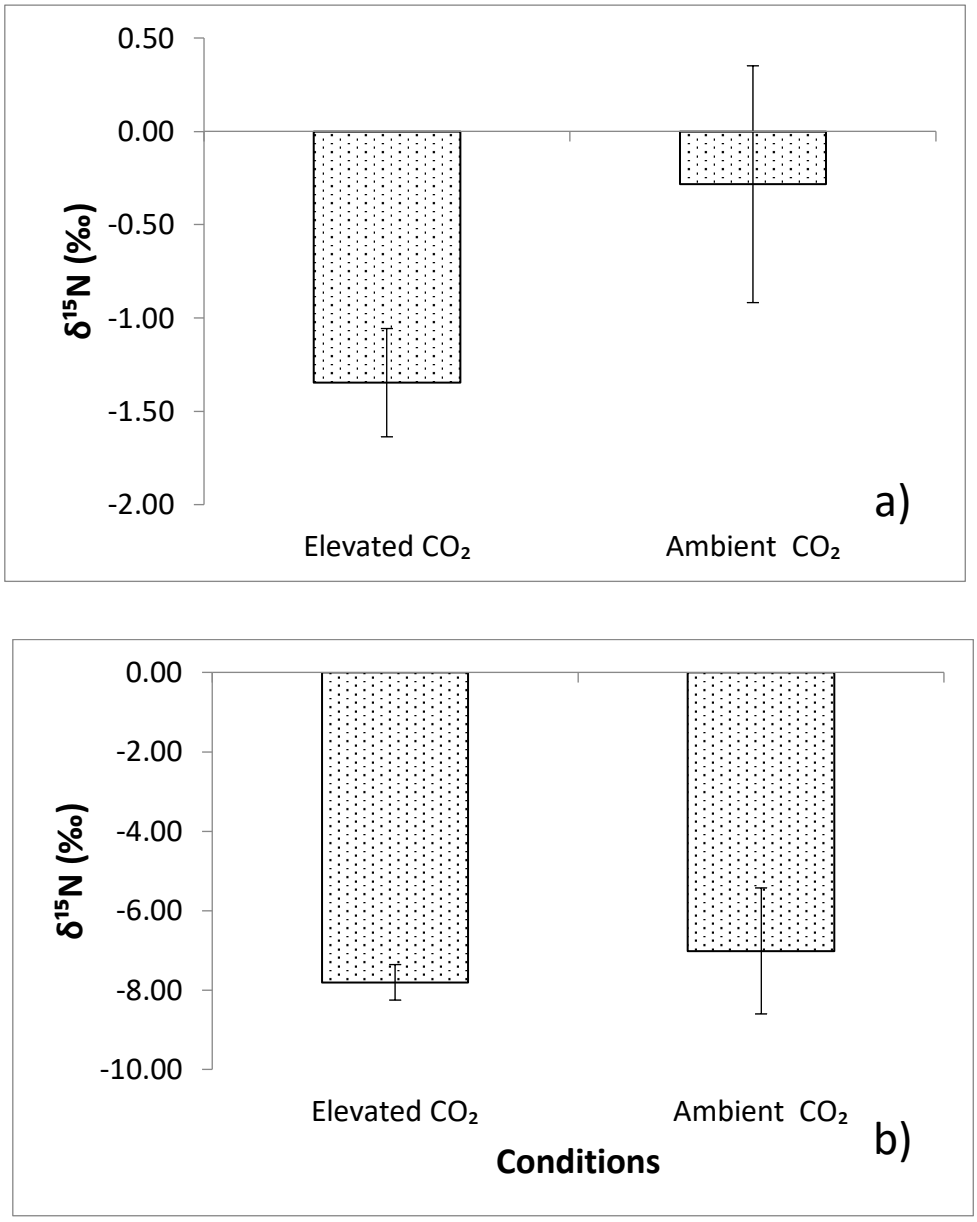

Figure 5.9. $\delta^{15}N$ natural abundance (in ‰) of the a) forest soil, and b) *H. cupressiforme*, under elevated CO_2 (550 ppm) and ambient CO_2 (n = 3). Data shown are means ±SE.

5.5 Discussion

These results showed that BNF activity in forest soil was stimulated under elevated CO_2 (Fig. 5.4a), going from 232 under ambient CO_2 to 1087 nmol gDW^{-1} d^{-1} under elevated CO_2, so that there would be an additional contribution of N that may support, to some extent, higher rates of NPP in future climate scenarios. This is in contrast to the results of several studies that found BNF activity unaffected by CO_2. One in a well-aerated soil of a temperate pine forest, the Duke Forest FACE site (Hofmockel and Schlesinger, 2007), and other in soil of a subtropical oak woodland (Hungate et al., 2014). However, in the former study site, the Duke Forest, the pines were planted on former agricultural land with a history of N fertilization legacy, and in the latter the results were based on a mesocosm experiment, using small young trees in chambers that were 9.42 m^2 and 2.5 m high. And both of these studies have used the acetylene reduction assay (ARA) method to measure the BNF rates which is an indirect method and ARA significantly underestimates BNF rates by more than 50% (Warren et al., 2017; Saiz et al., 2019) to accurately represent in situ BNF rates. Acetylene inhibits methanotrophy because it blocks the activity of methane monooxygenase enzyme and, as a result, bacteria are prevented from an important source of energy that otherwise could be used in the BNF process (Flett et al., 1975; De Bont and Mulder, 1976; Saiz et al., 2019). Recently, a study on methane oxidation found that ethylene, the result of the reduction of acetylene by the nitrogenase enzyme, may be the main inhibitor of the methane oxidation process, even at low concentrations (Bu et al., 2019). Temperate forest soils have an important community of methanotrophic bacteria, located primarily in the upper layers (Ullah and Moore, 2011), that play a key role as CH_4 sinks (Kolb et al., 2005), and most of the genera found in these soils are able to fix N_2 (Dedysh et al., 2004). In addition, acetylene also inhibits nitrification as well as the reduction of N_2O to N_2 in the denitrification process, but it does not affect the mineralization of N (Ryden, 1982), so all these changes would lead

to a decrease in the energy and substrate readily available for BNF. Apart from heterotrophic and methanotrophic bacteria, there are other diazotrophs to consider that fix N, phototrophs, although this group is less abundant in aerated soils than the others (Roper and Ladha, 1995), and these species interplay and affect each other (Larmola et al., 2014): CH_4 will feed methanotrophs and support their BNF activity, the resultant products will provide additional CO_2 to phototrophs and energy in the form of CH_2O to heterotrophs, they also may reduce the oxygen tension (less oxygen favour nitrogenase activity) and increase moisture. Therefore, phototrophs will benefit from CO_2 as well as heterotrophs and provide them with extra energy (CH_2O). Furthermore, it has been demonstrated that the availability of energy from microbial respiratory metabolites (CH_4, N_2O, and CO_2) increases BNF rates by more than 80% (Chapter 4). As a result, using the ARA method may cause a major disturbance in the biological community of the soil that even an increase in CO_2 will not fully reflect BNF rates (e.g. Hungate et al., 2014). These free-living diazotrophic bacteria are the most important source of new N in temperate forest soils (Levy-Booth and Winder, 2010), and they also are important contributors to N fixation in wetlands (Bae et al., 2018) and boreal forests (Mäkipää et al., 2018). The high increase in the BNF rates that was found could also have been facilitated by the particularly favourable conditions, regarding temperature, moisture, and light at the time the incubations were undertaken, enhancing methanotrophy and photosynthesis processes coinciding with a hot moment of BNF (McClain et al., 2003). Moreover, ARA method interferes with the activity of many microbes that fix N and thus previously reported results of BNF in temperate forest soils may not be realistic (Son, 2001; Tang et al., 2019). On the other hand, studies have shown that elevated CO_2 in forests reduces stomatal losses of water and thus slightly increases soil moisture (Warren et al., 2011). Under such conditions, it can be expected a high BNF activity as it was demonstrated by results on the forest soil (Fig. 5.4a).

Interestingly, BNF rates in *H. cupressiforme* were not stimulated under elevated CO_2. They decreased by 60% (Fig. 5.4b), from 3147 under ambient CO_2 to 1264 nmol gDW^{-1} yr^{-1} under elevated CO_2. These results are similar to the findings of a study about the BNF by epiphytic cyanobacteria on moss from a peatland under elevated CO_2 (Smith, 1984). But, in contrast to the findings of an experiment done with cultures of moss-associated cyanobacteria in the laboratory where BNF rates were higher under elevated CO_2 (Lindo and Griffith, 2017). However, besides the important decrease in the BNF rates, they were higher than the BNF rates of the soil samples that is in accordance with the general trend by which symbiotic BNF rates are higher than non-symbiotic ones (Son, 2001; Vitousek et al., 2002). The reasons for this decrease in the BNF rates may be twofold. In the first place, it could be because it has been demonstrated that the population of cyanobacteria associated with feather mosses decreases under elevated CO_2 (Steven et al., 2012), in fact, they reported a decrease of 59% that is really close to the value found here regarding the decrease of BNF rates (60%). Second, because of the difficulties in accessing to nutrients due to the boost in plant growth, and thus, an increase in nutrient consumption by the trees which is expressed by an improved nutrient use efficiency (Drake et al., 2011). The elevated CO_2 produces major changes in the forest ecosystem such as increasing NPP (Finzi et al., 2002), increasing litter inputs to soil (Zak et al., 2003), stimulating the rates of soil N mineralization (Hungate et al., 2014) due to an increase in microbial activity (Kuzyakov et al., 2019), all of which may increase the demand of other important nutrients. It was found that the concentrations of Mg, K, Co, and Ni in the soil samples were significantly lower under elevated CO_2 (Table 5.1) than under ambient CO_2, suggesting that in this case there was a greater consumption of these elements and thus reducing their availability from the soil. Soil nutrient availability is reflected in the bark of the tree (Gustafsson and Eriksson, 1995), so nutrients and cation exchange may be reduced, and it could be to a greater extent if the tree enters into efficiency

mode (Drake et al., 2011), fact that could affect the epiphytic *H. cupressiforme* and its associated cyanobacteria, resulting in lower BNF rates. There weren't significant differences in the concentration of P between elevated and ambient CO_2 conditions (Table 5.1), suggesting that P did not affect BNF activity in this case, because the addition of P enhances BNF as it was found in Chapter 4. However, this was just one BNF measurement at a specific point of time, and further measurements for a more detailed study would be needed.

The results of C and N content in *H. cupressiforme* were similar under elevated and ambient CO_2, resulting in very similar C:N ratios under both elevated and ambient CO_2 conditions that were 21 and 22 respectively (Fig. 5.5c). These results suggest that one year of CO_2 fumigation is not enough to see changes in C and N content as well as in C:N ratio. In addition, they could also be limited by the scarcity of other nutrients as indicated previously, and by environmental conditions. A study on bryophytes from a peatland carried out in the laboratory reported that C sequestration through photosynthesis under elevated CO_2 only increased during the first three days of the treatment after which it went back to control levels suggesting that C accumulation would only happen under favourable conditions for the mosses, this is, under availability of the nutrients needed (Van Der Heijden et al., 2000), as well as adequate temperature, moisture, and light (Rousk et al., 2013). In fact, moisture is an important factor controlling C and N fixation (Turetsky, 2003; Stewart et al., 2011), and there was no difference between CO_2 conditions (elevated and ambient) in *H. cupressiforme* (6.4 % on average in both). It was found a significant correlation between BNF rates and gravimetric moisture in *H. cupressiforme* (Fig. 5.6), and that gravimetric moisture in *H. cupressiforme* was significantly lower than in soil (Fig. 5.7). It has been reported that elevated CO_2 is likely to be less important than moisture or temperature as a factor affecting bryophytes (Lindo et al., 2013). Due to this important link between moisture and BNF, it is highly likely that future changes in precipitation and temperature patterns because of climate

change would affect BNF. In fact, it is expected a decrease in precipitation frequency and an increase in precipitation intensity which would lead to dry periods and floods (Dai et al., 2018). This scenario coupled with an increase in temperature because of global waming would significantly decrease soil moisture and increase evapotranspiration (Wang et al., 2016), that as a result would affect negatively BNF activity.

The C:N ratio of 2018 soil samples was significantly higher under elevated CO_2 than under ambient conditions. However, the C:N ratio of 2019 soil samples resulted not to be significantly different between elevated CO_2 and ambient conditions and in fact it was lower under elevated CO_2. The significant increase obtained in 2018 could be due to an anomaly, although other group of researchers working in the same plots at the same time (2018) obtained similar results (data not shown; personal communication). The results for both years (2018-2019) show a significant increase in C and N content under elevated CO_2. These results are limited. They were obtained after a year of fumigation with CO_2, and looking just at total C and N content, so further research looking at C sequestration and N uptake would be recommended for a better understanding.

It was found a significant difference in the natural abundance of $\delta^{15}N$ in *H. cupressiforme*, being higher under ambient CO_2 (Fig. 5.9b), reflecting that there was a higher supply of N from BNF activity under ambient CO_2, showing thus the decrease in BNF activity under elevated CO_2. However, the $\delta^{15}N$ signature was very low for *H. cupressiforme* under both CO_2 concentrations (Fig. 5.8), fact that may be because BiFOR FACE facility is surrounded by agricultural fields in which fertilizers are commonly used, and it is known that Nr from this source, that is transported by air (dry deposition) or by rain (wet deposition) giving a Nr deposition rate of 23 Kg N ha^{-1} yr^{-1}, has negative $\delta^{15}N$ values, and it is usually taken by plants, including epiphytic mosses, before reaching the ground (Kendall and Doctor, 2003). On the contrary, the $\delta^{15}N$ signature in the soil samples was closer to 0 (Fig. 5.8) suggesting

that the main source of N could be BNF activity (Deane-Coe and Sparks, 2016). Even though the $\delta^{15}N$ signature of the soil was slightly lower under elevated CO_2, the difference was not significant (Fig. 5.9a). However, this could suggest that other sources of nitrogen such as mineralization could increase under elevated CO_2. The difference between above ground mosses and forest soil $\delta^{15}N$ signatures may also be affected by the amount of Nr deposition that reaches the ground as well as by the way. The forest canopy may alter the amount of Nr deposition that reaches the ground and modify the way it reaches it (Wuyts et al., 2008; Cao et al., 2019). During rain events, epiphytic mosses would be affected by the stemflow that would reach the ground through tree trunks (Cao et al., 2019). There is also a gradient of N deposition reaching the forest floor from high rates in the border to lower rates in the interior which is called 'the edge effect' (Wuyts et al., 2008) that may have an important effect on BNF activity in forest soils.

At the ecosystem level, the contribution of *H. cupressiforme* to the N budget of the forest through BNF under ambient CO_2 was of 0.75 kg of N ha^{-1} yr^{-1} (the N load was estimated considering the extension covered by the feather moss in the study area as well as the growing season (Lindo et al., 2011) which is in line with the amount of N fixed by similar feather moss species in boreal forests (DeLuca et al., 2002). Under elevated CO_2 the rate was reduced to 0.20 kg of N ha^{-1} yr^{-1}, but it is still important due to the high rates of Nr deposition at the site (23 kg of N ha^{-1} yr^{-1}). On the other hand, the most active substrate that provides N to the system is the soil with rates that go from 22.85 kg of N ha^{-1} yr^{-1} under ambient CO_2 to 46.26 kg of N ha^{-1} yr^{-1} under elevated CO_2 (N load calculated considering the tree and understory coverage and the growing season). The amount of N entering the system doubled, this is an increase of 102% more. This result supports the idea that under elevated CO_2 the increase in NPP could be maintained, to some extent, because there is also an increase in new N. Some models have projected an increase in the total N demand by

36% (Finzi et al., 2002), which could be in part satisfied by the increase in BNF activity in the soil.

To conclude, just highlight the fact that it was found a significant increase on BNF in soil samples under elevated CO_2. On the other hand, it was found a decrease on BNF in *H. cupressiforme* under elevated CO_2 reflected as well in the $\delta^{15}N$ signature. This decrease also correlates with moss gravimetric moisture, and there was almost no difference in the C and N content of *H. cupressiforme*. These results suggest that temperate mature deciduous forests in future scenarios under elevated CO_2 and chronic atmospheric Nr deposition would double their new N source through BNF due to an increase in soil microbial activity. This study shows the effects just after a year of fumigation, so further research is needed to monitor these processes through time and confirm whether or not they follow the same trend.

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CHAPTER 6: SYNTHESIS AND CONCLUSIONS

6.1 Summary of key findings

There has been a massive increase in Nr availability due to the use of fertilizers and the burning of fossil fuels when, prior to the industrial revolution, the main sources of new N to the biosphere were through lighting and BNF (Vitousek et al., 2013). This anthropogenic Nr is altering the N cycle and causing environmental problems (Galloway et al., 2008). The UK has suffered high rates of atmospheric Nr deposition, and although the Nr emissions have been reduced during the last decades (Field et al., 2014), they are not synchronised with deposition and it will take longer for the Nr deposition rates to plateau and decline (Payne et al., 2017). In fact, it has been predicted still an increase by 2030 in areas with low Nr deposition rates such as the north of Scotland (Payne, 2014). Under such a scenario, the UK will still be above the Nr ecological threshold for many years to come. The main aim of this thesis was thus to evaluate the effects of increased atmospheric Nr deposition on rates of BNF in peatlands and temperate forests.

Summary and implications of Objective 1 outcomes

The first objective, outlined in the introduction, was to look for a strong method to measure BNF in the field. The most common method used to measure BNF was the ARA method (Table 1.3), however, it was indirect, and it needed a conversion factor to convert the amount of acetylene reduced into the amount of nitrogen fixed. The theoretical conversion factor was 3:1 (three moles of ethylene produce equal to one of N fixed; Hardy et al., 1968), however, it was highly variable, and because of it, many authors (e.g. Roskoski, 1981; Nohrstedt, 1983) recommended its calibration using the direct $^{15}N_2$ assimilation method for each location where it was going to be applied. These results showed that the conversion factor was extremely variable and far from the theoretical one. There were significant differences in the conversion factor between years in the same locations; among locations

and same species; and there were differences up to three orders of magnitude among species in the same location (Table 2.2). Furthermore, it was found that the ARA method underestimated BNF rates by 53% on average. This could be due to the presence of methanotrophs that can contribute up to 40% to total BNF in peatlands (Larmola et al., 2014; Vile et al., 2014). It was generally accepted that methanotrophy was inhibited by acetylene that deprives the organisms of energy essential to fuel the BNF process (Flett et al., 1975), however new findings indicate that it is ethylene (produce by the nitrogenase enzyme when acetylene is present) the gas that inhibits methanotrophy (Bu et al., 2019). Additionally, the presence of acetylene can interfere with other microbial processes and lead to a differential suppression of BNF. As a result, it was concluded that ARA method is not adequate to measure BNF activity in peatlands and it is strongly recommended, thus, the robust and direct $^{15}N_2$ assimilation method.

These results imply that the BNF rates of studies in which the ARA method was used to measure BNF in *Sphagnum* mosses and peat could be underestimated. In addition, the use of the ARA method could affect the BNF rates in other ecosystems where N_2-fixer microbes are affected either by acetylene or ethylene. These underestimations could have led to miscalculations of the nitrogen budget of these ecosystems, and it could be the reason why some studies mentioned that there was a unaccounted source in the inputs of the N budget of some ecosystems (Son, 2001).

Summary and implication of Objective 2 outcomes

In relation to the second objective regarding the effects of Nr deposition on BNF and its controls it was found that chronic high rates of Nr deposition (>26 kg N ha^{-1} yr^{-1}) did not shut down BNF. These results showed that the effects of BNF suppression were higher in areas with lower Nr deposition rates, and they decreased following a power equation as Nr

deposition rates increased. This suggests the microbial community of N_2 fixers is resilient and they can adapt to different levels of Nr deposition. The results also showed that species in hollows fixed 61% more N than species in hummocks, suggesting than landscape topography may play a crucial role in combination with other factors: the first one, hollow areas contain more moisture than hummocks, and it has been demonstrated that BNF increases with moisture (Rousk et al., 2018); second, in hollows there may be a higher availability of mineral nutrients (Stewart et al., 2011); and third, hummock areas receive a more direct Nr deposition than hollows where Nr deposition may be directly diluted in water having a lesser effect on BNF. It was not found any significant correlation between BNF and some of the main abiotic factors such as temperature, pH, light, moisture, electrical conductivity or dissolved oxygen. Suggesting, thus, that direct measurements of BNF in the field may be affected by complex interactions of all these abiotic factors at the same time, not being able to detect any relationship in isolation but considering all of them at once.

One of the implications of these results is that authors working on the modelling of peatlands that use data about the nitrogen budget and assume that there is no BNF under high rates of Nr deposition (> 10 kg N ha-1 yr-1) may miscalculate N inputs and thus lead to wrong conclusions (Heijmans et al., 2008; Pullens et al., 2017). On the long term, even a low BNF activity under high rates of atmospheric Nr deposition would be an extra source of N that would make it more readily available to vascular plants. These vascular plants, with time, may overtake mosses in peatland ecosystems. Under these circumstances plant diversity in peatlands would decrease because of an increase of nitrophilous vascular plants (Levy et al., 2019). At the same time, there would be a change in the N biogeochemistry of peatlands where BNF would decrease, decomposition rates would increase (increasing the availability of organic N) as well as denitrification rates, which would imply peatlands becoming N sources, in fact, sources of one of the greenhouse gasses N_2O responsible for climate change.

To compound the problem, climate change would also pose a threat to BNF activity and to peatlands. Temperature and precipitation are two of the main controls of BNF that counteract each other in the field and would change for worse due to an increase in temperatures (increasing evapotranspiration and decreasing moisture) and to a decrease in precipitation frequency and an increase in intensity (increasing runoff and dry periods; Dai et al., 2018). This scenario would lead to the disappearance of peatlands due to changes in vegetation and water table and to a decrease in BNF activity in other ecosystems, e.g. temperate forests where a decrease in moisture, even with increases in temperature, would result in a decrease in BNF activity.

Summary and implications of Objective 3 outcomes

Regarding the effects of some macro and micronutrients on BNF in the field, it was found, in a peatland in northern Sweden, that decades of Nr treatment did suppress BNF, but that it was the combination of N, S and T what affected BNF the most suppressing it to very low rates. However, BNF did not shut down. In the laboratory using *Sphagnum* mosses and peat, the results of an experiment under Nr saturation showed that the addition of K and of P independently increased BNF but, that it was the addition of these elements combined, what had a higher boosting effect on BNF. This demonstrates that under no N limitation, P & K play a co-limiting role in BNF activity as it has been found for *Sphagnum* plants growth (Bragazza et al., 2004). Furthermore, when N, P, and K are no longer limiting nutrients it was found that the addition of Mg and of MRMs increased BNF considerably. Suggesting that under the availability of N, P, and K the main limiting factors were C in the form of energy obtained from the reduction of N_2O, oxidation of CH_4, and photosynthesis (CO_2).

These results provide us with a better understanding of the effects of nutrients and micronutrients on BNF. The results show that the microbial processes are connected and that

they work as in a factory where you need a set of specific supplies (components and energy) to terminate a product, and if one of them fails (it is not provided) the product cannot be terminated whatever the surplus of other components the factory has. This knowledge is of great importance in the area of forest management, because some forested areas, for example in Sweden, that are N limited have been fertilized with high amounts of N, and additionally with P, but they have not got the desired results (Binkley and Högberg, 1997). This lack of results is possibly because of an excess in one of the elements, and thus a limitation in others such as P or K. At this respect, knowing that under Nr saturation the addition of other essential nutrients and micronutrients boost microbial processes would be critical to optimise these fertilization campaigns in forests and thus increase the health and productivity of the forests. In addition, the results obtained in the laboratory also suggest that in wet soils, where it could be an increase in the production of MRMs (CH_4, N_2O, and CO_2), an increase in the concentration of MRMs in the porewater and poreair would also enhance BNF activity under certain conditions. So, the present increase trends in the concentration of these gasses would pose an extra surplus of N which should be considered by forest managers for optimum production.

Summary and implications of Objective 4 outcomes

Assessing the effects of elevated CO_2 on BNF in a temperate mature forest, two different results were found. On the one hand, the BNF activity in epiphytic *Hypnum cupressiforme* mosses was reduced under elevated CO_2. This result could be linked to the fact that the main type of N_2 fixing bacteria associated with *H. cupressiforme* is cyanobacteria, and its population also decreased in similar terms under elevated CO_2 (Steven et al., 2012). Moreover, this result suggests that epiphytic mosses activity may be limited by other factors such as the reduction of availability of macro and micronutrients due to an increase in tree nutrient use efficiency that is reflected in the bark (Gustafsson and Eriksson, 1995), which

would be related to Objective 3 outcomes. On the other hand, forest soil (0-5 cm) non-symbiotic BNF was increased massively. This high increase of BNF could be due to an increase of N demand under elevated CO_2 and thus a competition for N in soils by trees and microbes that may have forced the microbes to seek N through BNF. C and N availability is also reflected in the soil C and N content that was significantly higher under elevated CO_2 than under ambient CO_2.

These results demonstrate how BNF is connected to the availability of nutrients, energy, and N demand. The implications of these findings are of great importance regarding climate change and the increase of greenhouse gasses. On the one hand, under an increase in CO_2, these results show that there was an increase in BNF in the forest organic soil, and therefore an increase in N supply. This increase in BNF is critical as the demand for N by plants would also increase due to the surplus of CO_2. In fact, it has been suggested that NPP in forests will not be able to respond to an increase in CO_2 (and increase plant growth) if the there is no increase in other limiting nutrients such as N (Finzi et al., 2006), and it has been demonstrated that there was an increase in N supply that would support to some extent an increase in forest NPP.

General conclusion

It can be concluded that increased atmospheric Nr deposition reduces BNF activity but that it does not shut it down; and that under Nr saturation BNF is enhanced by the addition of other key limiting macronutrients such as P and K. In addition, BNF activity under no limitation of Nr, P, and K is boosted by the addition of key micronutrients in the BNF process such as Mg, and MRMs (microbial respiratory metabolites: CO_2, CH_4, and N_2O; Fig. 6.1).

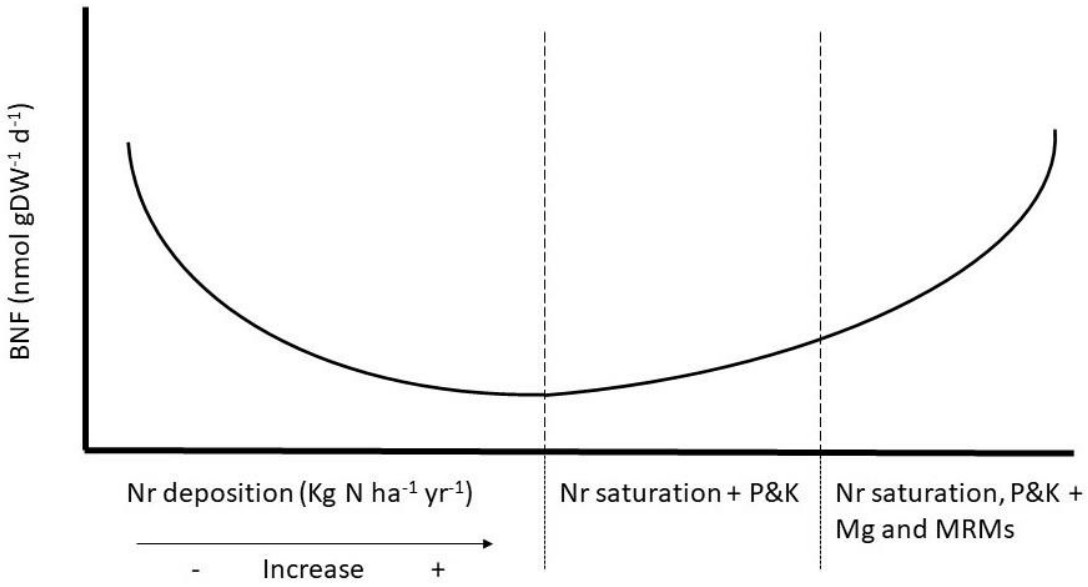

Figure 6.1. A conceptual model for the effects of Nr deposition and the addition of macro and micronutrients and microbial respiratory metabolites (MRMs) on BNF. The x-axis represents Nr deposition as the amount of Nr per area and time (e.g. Kg N ha^{-1} yr^{-1}) in the first section which increases from left to right. The next section in the x-axis divided by a dotted line represents the addition of P and K under Nr saturation. And the final section (divided by the dotted line) in the x-axis represents the addition of Mg and MRMs (CO_2, CH_4, and N_2O) under Nr saturation and no limitation of P and K. The y-axis represents BNF activity in nmol gDW^{-1} d^{-1}. (Source: prepared by the author)

6.2 Further research

The work in this thesis has addressed some specific objectives regarding the effects of high rates of Nr on BNF in peatlands and forests, but at the same time, it has raised new questions and aspects for further investigation. For example, the abiotic factors affecting BNF in temperate peatlands remain unclear, and a comprehensive study looking at how they interact with each other and their effects on BNF under field conditions is recommended. It has been found, in this research, that there was no correlation between abiotic factors such as pH, EC, temperature, and moisture in the field, even though that it has been demonstrated in the laboratory that they are controlling factors of the nitrogenase enzyme. In addition, there is evidence of different types of bacteria being host by *Sphagnum* mosses depending on these

controlling factors (Opelt et al., 2007; Leppänen et al., 2015; Carrel et al., 2019). Therefore, further research, field-based, would be needed to investigate how changes in these controlling factors (pH, EC, temperature, and moisture) are linked to the types of N_2-fixing bacteria in *Sphagnum* mosses and all the controlling factors alone and in combination and the types of bacteria to BNF activity, in the long term.

In this research it has been found that BNF activity under Nr saturation is boosted by the addition of nutrients such as P and K, micronutrients such as Mg, or MRMs such as CO_2, CH_4, and N_2O. In short, that BNF was limited by macro and micronutrients or energy. However, these results were only for short-term responses and they were from experiments carried out in the laboratory, so to evaluate if this elevated BNF activity can be maintained in the field, and if long-term fertilization will support vascular plants to overtake mosses, further research carried out in the field would be needed.

More research is also needed to investigate how an increase in CO_2 would affect BNF in mature temperate forests. These results show the effects of elevated CO_2 after a year of treatment in which BNF under elevated CO_2 decreased in epiphytic mosses aboveground while BNF increased in forest organic soil layer (0-5 cm). However, they are just one measurement at one point of time, and to evaluate if this is the general trend or just a one-off result it is recommended a long-term study (several years) monitoring BNF activity during the growing season, as well as looking at the main controls affecting it (pH, nutrients, temperature, precipitation, etc).

In addition, it was examined the BNF in a mature deciduous forest that has been exposed to chronic rates of Nr deposition for decades and the rates were high, so it would be needed further research looking at mature deciduous forests with low Nr deposition to compare BNF activity in this type of forests under an Nr deposition gradient.

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